Cytomegalovirus and chronic rejection of liver grafts

Thesis

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CYTOMEGALOVIRUS AND CHRONIC REJECTION OF LIVER GRAFTS

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A thesis submitted in partial fulfillment of the requirements of the Open University for the degree of Doctor of Philosophy

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DEPARTMENT OF MEDICINE, UNIVERSITY OF CAMBRIDGE SCHOOL OF CLINICAL MEDICINE, CAMBRIDGE, UK

ADDENBROOKE'S NHS TRUST
ABSTRACT

This thesis tested the hypothesis that cytomegalovirus (CMV) may initiate or enhance chronic rejection of liver grafts. A polymerase chain reaction (PCR) test was developed and used to monitor 33 liver transplant patients for active CMV infection (Chapters 2 and 3). The incidence of urine PCR positivity and prolonged active CMV infection were risk factors for chronic rejection (Chapter 4).

Matching and mismatching of HLA alleles between donor and recipient was not shown to be a risk factor for chronic rejection. However, HLA class I matching was associated with clearance of active CMV (Chapter 4).

Recipients with the TNF-2 promoter allele (associated with enhanced expression of TNF) were at increased risk of chronic rejection. Furthermore, active CMV infection and the TNF-2 promoter allele were shown to act synergistically as risk factors for chronic rejection (Chapter 4).

In addition, two or more episodes of acute rejection or a pre transplant diagnosis of primary biliary cirrhosis (PBC) were shown to be risk factors for chronic rejection (Chapter 4).

CMV was also studied in the context of humoral immunity and chronic rejection (Chapter 5). Western blotting of hepatic artery and bile duct tissue (sites of rejection mediated damage) showed that post transplant IgA antibody to a 44 kD bile duct protein was associated with development of active CMV infection but was not associated with chronic rejection. However, pre transplant IgA antibodies to 94 and 39 kD bile duct proteins or IgG antibodies to 160 and 85 kD hepatic artery proteins were associated with an increased risk of chronic rejection.

CMV was identified in bile duct epithelial cells, vascular endothelial cells, hepatocytes and mononuclear cells of liver grafts by in situ hybridisation (Chapter 6). Active CMV infection of the graft, especially epithelial cells, was associated with chronic rejection.

Finally, human herpesvirus 6 (HHV-6) was not detected in serum, whole blood or liver graft tissue by PCR and was not an important pathogen after liver transplantation.
Acknowledgements

I acknowledge and thank Dr Graeme Alexander (Director of Studies) for providing superb advice and direction over the past three years, for patience during times of 'technical crisis' and for encouragement and support. Similarly, the advice of Dr Tim Wreghitt (Second Supervisor) has been crucial to this thesis.

I also gratefully acknowledge Dr Jane Collier, Mr Avi Soin and Mr Giles Toogood for provision of clinical data, Dr Nick Coleman and Miss Alison Warn for provision of liver sections and protocols for in situ hybridisation, Dr Craig Taylor and Miss Sheila Smith for provision of protocols for HLA typing and TNF allele PCR and advice on the interpretation of HLA data, Dr Brian Thompson and Mrs Chen Huang for provision of reagents and protocols for HHV-6 detection and Dr Jim Gray for provision of reagents and protocols for detection of CMV by DEAFF and culture. I am indebted to other colleagues who are acknowledged throughout this thesis.

I acknowledge the financial support provided by the Anglia and Oxford Health Authority.

The encouragement provided by my colleagues in Dr Alexander's laboratory is also greatly appreciated; this 'dream team' comprises Simona Caronia, Shaun Greer, Lesley Ramsay and Shim Wong.

Finally, I thank my fiancee, family and friends for their support.
DECLARATION

The work presented in this thesis was conducted by the author, unless otherwise acknowledged.

Signed

Paul Evans
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<td>170</td>
</tr>
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CHAPTER 1

INTRODUCTION
1.1 A Historical Account of Cytomegalovirus

'Protozoan like' cells were initially observed in the kidney of a newborn child in 1881 by Ribbert (1). Similarly, Jesionek and Kiolemenoglou (1904) (2) published a case report describing large (20-30μm diameter) cells found in kidney, lung and liver of an eight month old fetus; intranuclear inclusions were observed in these cells.

The cells described in these early case reports of congenital disease displayed the hallmarks of CMV infection; indeed, an infectious agent was thought to be responsible but initially this was thought to be an amoebiform protozoan. Smith and Weidman (1910) (3) proposed the name Entamoeba mortinatalium.

In the 1920's a viral aetiology was suspected. Goodpasture and Talbot (1921) (4) described a six week old infant; cells found in kidney, lung and liver were described and termed "cytomegalic" for the first time. This paper was also a milestone in the history of cytomegalovirus because the authors proposed that the intranuclear inclusions seen were similar to those seen after varicella infection and doubted protozoan involvement.

Von Glahn and Pappenheimer (1925) (5) also proposed a viral aetiology and suggested membership of the herpesvirus group; analogies were made between 'cytomegalic inclusions' and inclusions associated with herpes zoster and herpes simplex.

Survival of an infected neonate was reported by Margileth (1955) (6). Clinical symptoms at birth included hepatosplenomegaly, haemolytic
anaemia, thrombocytopenia and microcephaly. In addition, a diagnosis of cytomegalic inclusion disease was confirmed after detection of cytomegalic cells in urine (the first reported method of laboratory diagnosis).

Isolation of this suspected virus was achieved after the advent of cell culture in the 1950's. Smith (1956) (7) described isolation from samples of salivary gland and kidney, taken from infants with cytomegalic inclusion disease. Viral particles replicated in human uterine cell cultures.

In addition, two groups isolated CMV by accident. Rowe et al (1956) (8) and Weller et al (1957) (9) were attempting to isolate adenovirus and *Toxoplasma gondii* respectively. Inclusions in cell cultures of human adenoid tissue (8) and skin-muscle tissue (9) were reminiscent of those seen after cytomegalic inclusion disease.

In modern times, the study of cytomegalovirus (CMV) has changed focus dramatically; immunosuppressed individuals i.e. organ transplant recipients receiving immunosuppressive drugs and patients with acquired immune-deficiency syndrome (AIDS) are new populations at risk of severe CMV disease.

The advent of molecular biology has provided powerful tools which have enabled a better understanding of the biology of CMV.
1.2 Cytomegalovirus is a Member of the Human Herpesviridae

1.2.1 Members

The human herpesviridae group consists of eight DNA viruses namely herpes simplex virus type 1 (HSV 1), herpes simplex virus type 2 (HSV 2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), human herpesvirus 6 (HHV 6), HHV 7 and HHV 8. The molecular and biological characteristics of the latter two recently described viruses are poorly understood.

The herpesviruses share a number of characteristics that justify their grouping. The genomes are large (over 100 Kb), double-stranded, linear DNA molecules. The genomes are complex; HSV and CMV genomes encode 72 and 208 open reading frames respectively (10, 12). The genetic material is contained within an icosahedral shaped capsid which is surrounded by an amorphous proteinaceous tegument and finally, a lipid membrane envelope (10). An overall molecular weight of $10^8$ Daltons is similar for all human herpesviruses (11).

In addition, a number of homologies between DNA and protein sequences have been found throughout the human herpesviruses (10, 13, 14, 15, 16).

Herpesviridae have been delineated into 3 sub-groups on the basis of tropism: alpha (α), beta (β) and gamma (γ)-herpesviridae (17). α-herpesviridae include HSV-1, HSV-2 and VZV and are neurotropic whereas β-herpesviridae, which include CMV, and γ-herpesviridae, which include EBV, are lymphotrophic. Members of each sub-group also have similar genome structures and other biological characteristics (10, 17).
1.2.2 Prevalence

Herpes viruses are prevalent; in Britain 50-60% and over 90% of the adult population are chronic carriers of CMV and EBV respectively (18, 19). The prevalence of the herpes simplex viruses (HSV) varies worldwide, depending upon socioeconomic factors, approaching 100% in some populations (20). Similarly, HHV-6 is highly prevalent and infection rates between 50-95% have been described (33).

The seroprevalence of CMV antibodies increases with age; in a Swedish population seroprevalence increased from 19% (age 1-3 months) to 53% (age over 50 years) (76). Seroconversion rate, which is a reflection of infection rate, peaked between 6 months and 5 years.

1.2.3 Infection of Immunocompetent Individuals

Upon primary infection an individual may display a spectrum of well recognised symptoms, e.g. infectious mononucleosis (EBV), oro-facial or genital lesions (HSV 1). However, herpesviruses often infect asymptotically or give rise to unrecognised symptoms. Primary infections with CMV, EBV or HSV in healthy individuals are rarely symptomatic, or symptoms are not recognised (85) but less than 4% of VZV primary infections in children are inapparent (21).

After primary infection, herpesviruses persist latently in a restricted population of host cells. Reactivation of the latent population occurs sporadically and this may lead to a symptomatic infection, but in a healthy individual viral replication is often ablated efficiently by specific cellular and humoral immune responses (see section 1.4). Thus a large proportion
of reactivation infections are asymptomatic, e.g. 50% of HSV-1, reactivations have been shown to not manifest clinically (22).

1.2.4 Infection of Immunosuppressed Individuals
In an immunosuppressed individual, viral replication is not suppressed if T cell immunity is impaired; this may lead to severe symptomatic infection. High risk groups for symptomatic herpesvirus infection include neonates (80, 81), patients with AIDS (82, 83, 84, 90) and transplant recipients.

EBV is an important pathogen after liver (23, 24), bone marrow (27, 30), renal (25, 26) and cardiac (38, 29) transplantation. Similarly, active VZV and HSV infections have been recorded after bone marrow transplantation ((31, 32, 33, 34; VZV), (32, 33, 34; HSV)) or liver transplantation ((32; HSV), (34, 35; VZV)).

The importance of CMV infection in transplant patients is covered in sections 1.5, 1.6, 1.8 and 1.9.

1.3 Virology and Biology of Cytomegalovirus

1.3.1 Genes
Cytomegalovirus (CMV) (strain AD169) was sequenced by Chee et al (1990) (12). This large and complex genome is present as a linear, double stranded molecule within the cell nucleus; it is 230 Kb in size and encodes 208 open reading frames.

The genome comprises a long unique sequence (UL) and a short unique sequence (US); each is flanked by inverted repeats (IR). The unique
sequences can invert during replication to produce 1 of 4 possible isomers (59).

Homology has been found between CMV DNA and human DNA and/or DNA from other herpesviruses. This is shown diagramatically in figure 1.1.
Figure 1.1

(a) Structure of the CMV genome; the prototypic isomer is shown.

(b) Eco R1 restriction map of the CMV genome; the major fragments are labelled and labels reflect fragment size (A largest, Z shortest).

(c) Hind III restriction map of the CMV genome; the major fragments are labelled and labels reflect fragment size (A largest, Z shortest).

(d) Regions of the CMV genome with homology to CMV; based upon hybridisation of Eco R1 restriction fragments (Shaw et al. [130])

(e) Regions of the CMV genome with homology to fragments (Ruger et al. [173])

TRL = long segment terminal repeats, IRL = long segment internal repeats, TRS = short segment terminal repeats, IRS = short segment internal repeats, UL = unique long region, US = unique short region.
1.3.2 Latent Infection

The ability to establish latent infection is a characteristic shared by all herpesviridae.

Herpes simplex virus (HSV) latently infects neural ganglia; in this state, HSV produces a transcript known as LAT (latency associated transcript). LAT is not translated but is thought to be important in maintaining a latent state (36). Epstein Barr virus latently infects epithelial cells (37) and B-lymphocytes (38).

Historically, epidemiological evidence from the study of seroconversion after blood transfusion suggested that blood cells were a site of latency for CMV (39, 40). However, demonstration of latent CMV in monocytes has only recently been achieved after implementing an extremely sensitive detection technique i.e. nested PCR (41). Furthermore, bone marrow progenitors of the monocyte/macrophage lineage have been shown to harbour latent CMV (42).

The epidemiology of CMV infection after solid organ transplantation strongly suggests that the donated organ harbours latent CMV (43). Attempts to isolate CMV from the tissue of healthy CMV antibody positive individuals has been unrewarding. However, in a recent report murine CMV (mCMV) was found to be tropic to endothelial cells of lung, liver and spleen (44). Here, in situ-PCR technology was used which allowed highly sensitive detection of mCMV whilst preserving cellular architecture and therefore allowing identification of latently infected cells. This finding has not been reported for human CMV.
Lungs are an important site of pathology during active CMV infection (109) and it has been suggested that alveolar macrophages harbour latent CMV (45). Balthesen et al (1993) (45) infected BALB/c mice with $10^2$ pfu murine CMV (mCMV), 1 day after birth. In surviving mice (60%), replicating virus was found in salivary glands for up to 6 weeks after infection. Latency was established after this time; replicating virus was not cultured from salivary gland, lung or spleen at 1 year post-infection. However, PCR amplification demonstrated latent mCMV in lung and spleen.

Latency in lung tissue has not been properly investigated for human CMV; Fajac et al (1994) (46) could not detect latent CMV in bronchoalveolar lavage from healthy CMV antibody positive individuals after PCR. The authors conceded that this may be due to lack of sensitivity; latent CMV was also undetected in blood mononuclear cells which have been shown to be a latent site by others (41, 42).

To date, a 'latency associated transcript' has not been found for CMV. Studies conducted in the Department of Medicine, University of Cambridge by Dr. Sinclair demonstrated that host-factors are important. Specifically, this group showed that monocytes (cell line THP 1) were non-permissive for productive CMV infection because the major immediate promoter-enhancer (12) was repressed (also see below). Immediate early expression was restored \textit{in vitro} after differentiation of monocytes into macrophages (48). In a model system, non-permissive tetracarcinoma (T2) cells were differentiated after addition of retinoic acid; YY1 (a host transcription factor) was lost from regulatory elements positioned within and upstream of the major immediate early promoter-enhancer and this
1.3.3 Lytic Infection

A 'cascade' of transcription occurs after the initiation of lytic infection. CMV genes have been designated immediate early (IE), early (E) or late (L) according to the time of transcription initiation.

1.3.3.1 Immediate Early Genes

Immediate early (IE) genes 1 and 2 are both controlled by the major immediate early promoter. Both are transcribed immediately after and throughout infection (50). Cyclohexamide (translation inhibitor) does not prevent IE gene transcription which probably occurs using host cell RNA polymerase (51).

Immediate early mRNA is translated in the cytoplasm; IE proteins are transported into the nucleus where they function as transcription factors (52).


IE genes are also vital for transcriptional activation of the early (E) and late (L) genes (55).
1.3.3.2 Early (E) Genes

Early genes are transcribed 2-24 hours after infection; generally, these genes are not structural but encode a number of enzymes crucial to assembly of intact virions. Early gene products include DNA polymerase (140kD) and DNA binding proteins (52 and 140kD) (52). Other gene products have been predicted from DNA sequence analysis including a DNase, helicase, deoxy-UTPase, uracil-DNA glycosylase and helicase-primase (56).

1.3.3.3 Late Genes

Late gene and early gene transcripts are produced simultaneously; however, late gene transcripts are retained in the nucleus until viral DNA synthesis is initiated at 12 hours post-infection (57). Therefore, late gene products appear after 24 hours; the mechanism of retention is unclear.

Late genes (L) are predominantly structural; their function within the virion is shown in figure 1.2.

1.3.4 Effect of CMV Infection on Cell Protein Contents

CMV infection leads to production of CMV proteins and altered host cell protein synthesis. This leads to an altered immune response which has important implications for the study of the relationship between CMV and allograft rejection. For example, humoral or cellular responses to CMV proteins or upregulated auto-antigens may occur (see sections 1.3.3.1 and 1.4).
Figure 1.2

Schematic of the CMV virion: an icosahedral structure

**Nucleocapsid**

Major nucleocapsid protein 150 kD (90% whole nucleocapsid)
- gp34 34 kD
- gp28 28 kD

**Matrix**
- Lower matrix protein (pp65) 65 kD
- Upper matrix protein 71 kD
- Basic phosphoprotein 150 kD

**Envelope**

Double lipid bilayer with glycoproteins:
- gB 55/92 kD complex
- gH 86 kD
- gC II 50/>200 kD complex
- gp48 48 kD

**CMV genome**
Linear 230 Kb molecule
1.3.5 CMV Encodes a Protein with Homology to the MHC Molecule
Beck and Barrell (1988) (60) performed DNA sequence analysis upon the human CMV gene H301. From a predicted protein sequence it was shown that the H301 gene product has 20% homology with the HLA class I gene. The β2 microglobulin (β2-m) binding site is a loop of 6 amino acids present in the A-3 domain of class I; in the CMV homologue 4/6 amino acids were shown to be conserved. In addition, the CMV homologue was shown to contain a conserved region of 23 hydrophobic amino acid residues responsible for spanning the cell membrane.

CMV proteins were shown to bind β2-m in vitro by Grundy et al 1987 (61); two proteins (36 and 65 kD) were identified. Beck and Barrell (1988) (60) speculate that, depending upon the extent of glycosylation, the H301 gene product may assume a molecular weight of 65kD in vivo.

CMV virions were found coated with β2-m in vivo by McKeating et al 1987 (62). Here, the authors suggested that CMV virions exchange their surface bound β2-m for class I associated β2-m to facilitate cell entry after class I/β2-m/CMV complex endocytosis.

1.4 Immunity and Cytomegalovirus

1.4.1 Cellular Responses

1.4.1.1 Cytotoxic T Lymphocytes (CD8 +)
Autologous antigen presenting cells and T-lymphocytes can be combined to investigate MHC restricted T-lymphocyte responses; lysis of target cells is measured by $^{51}$Cr release. This system was used by Borysiewicz et al (1983) (63); bulk cultures of peripheral blood mononuclear cells from
healthy CMV antibody positive individuals contained cytotoxic lymphocytes (CTL) (CD8+) that responded specifically to CMV antigens. In a subsequent paper, Borysiewicz et al (1988) (64) used limiting dilution assay to investigate the proportion of CTL precursors having specificity against IE-1 and gB proteins expressed in vaccinia-vectors in autologous fibroblasts; 20 - 60% clones recognised IE-1 whereas less than 4% recognised gB.

In contrast, Gilbert et al (1993) (367) studied MHC restricted presentation of CMV proteins after active CMV infection of fibroblasts (as opposed to vaccinia construct expression of individual CMV proteins) and found that the proportion of IE protein specific T-lymphocytes (after stimulation of peripheral blood mononuclear cells with CMV infected fibroblasts) was low.

This was due to inefficient presentation of IE peptides by CMV infected fibroblasts; T-lymphocytes with specificity to pp65, gB and IE led to specific lysis of 51%, 25% and 2% of CMV infected target cells respectively. It was proposed that this mechanism allows CMV to escape detection of IE protein. Further reports showed that MHC class I heavy chain/IE peptide complexes were rendered unstable (253) by the activity of early genes (252).

McLaughlin-Taylor et al (1994) (430) have shown that pp65 is a common target for CD8+ class I MHC restricted cytotoxic lymphocyte activity amongst latently infected individuals. Studies of transplant patients by one group suggest that cellular responses to CMV after transplantation may protect against CMV disease (65, 66). Quinnan et al (1982) (65) studied 58 bone marrow transplant recipients; 43 developed CMV infection
including 28 cases of CMV pneumonitis. All 18 patients that survived developed CMV-specific cellular responses (9 CTL and 9 natural killer cells (NK)) whereas only 2 of 10 patients that died developed an NK and none developed a CTL response. However, cellular responses were not developed by patients without CMV disease suggesting that other immune factors are involved. In addition, Grundy et al (1987) (67) suggest that CMV pneumonitis is immunopathogenic (see section 1.6.6.4).

1.4.1.2 CD4+ T-Lymphocytes
Fewer studies have focused on the role of CD4+ T-lymphocytes. These cells were collected from CMV antibody positive individuals and were shown to be cytotoxic towards autologous monocytes (which express MHC class II) expressing CMV protein (Lindsay 1986) (68). Cytotoxic CD4+ T-lymphocytes have also been shown for mCMV. Shanley et al (1987) (377) performed adoptive transfer of CD4+ and CD8+ T-lymphocytes from mice which were latently infected with mCMV into naive, homozygous nude mice (deficient in mature lymphocytes) before infection with mCMV (21 days later); only transfer of CD4+ T-lymphocytes suppressed viral replication. Furthermore, Geier SA (1995) (378) found reduced CD4+ T-lymphocyte counts to be an important prognostic factor for active CMV infection in patients with AIDS.

Interferon-γ production production by CMV specific CD4+ T cells has also been shown (431, 432). Waldman et al (1993) (431) suggested that such IFN-γ production may lead to proliferation of alloreactive T cells in addition to CMV specific T cells and suggest that CMV may contribute to graft rejection indirectly.

1.4.2 Humoral Immunity
Antibodies reactive with specific CMV proteins have been found in CMV antibody positive individuals; target proteins are listed in table 1.1.
Table 1.1
Proteins identified as targets for antibody production in CMV antibody positive individuals.

<table>
<thead>
<tr>
<th>NAME</th>
<th>MOLECULAR WEIGHT</th>
<th>VIRION LOCATION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-binding protein</td>
<td>52 KDa</td>
<td>NA</td>
<td>Gergely et al 1988 (7)</td>
</tr>
<tr>
<td>DNA-binding protein</td>
<td>35 KDa</td>
<td>NA</td>
<td>Gergely et al 1988 (7)</td>
</tr>
<tr>
<td>Structural Protein</td>
<td>28 KDa</td>
<td>Capsid</td>
<td>Re et al 1985 (8)</td>
</tr>
<tr>
<td>Lower matrix protein</td>
<td>64 kDa</td>
<td>Matrix</td>
<td>Forman et al 1985 (9)</td>
</tr>
<tr>
<td>Basic phosphoprotein</td>
<td>150 KDa</td>
<td>Matrix</td>
<td>Landini et al 1986 (10)</td>
</tr>
<tr>
<td>Glycoprotein gC II</td>
<td>52 / &gt;200 KDa</td>
<td>Envelope</td>
<td>Liu et al 1988 (11)</td>
</tr>
<tr>
<td>Glycoprotein gB</td>
<td>92 / 55 KDa</td>
<td>Envelope</td>
<td>Liu et al 1988 (11)</td>
</tr>
<tr>
<td>Glycoprotein gH</td>
<td>86 KDa</td>
<td>Envelope</td>
<td>Liu et al 1988 (11)</td>
</tr>
</tbody>
</table>

*Commercial antigenaemia assays contain antibody-conjugates that are directed to the 65 kD lower matrix protein (379).

Antibodies reactive with CMV glycoproteins have been shown to be neutralising in vitro but this has not been shown in vivo (74). The presence of CMV-reactive antibodies in transplant recipients does not necessarily prevent infection but may reduce the severity of CMV disease (75).
1.5 Liver Transplantation and Cytomegalovirus (CMV)

1.5.1 Immunosuppression Facilitates Active CMV Infection
Liver transplantation is the treatment of choice for most end-stage liver diseases. Immunosuppressive drugs minimise the incidence of rejection by reducing the recipients ability to mount a cellular immune response against the graft, e.g. cyclosporine abrogates the transcription of cytokine mRNA in activated lymphocytes. When first introduced, cyclosporine-A improved renal graft survival rates at one year by 20% (101). However, it has been shown by Converse et al 1983 (97) that cyclosporine reduces the response of cytotoxic lymphocytes to CMV infected fibroblasts in vitro.

Active CMV infection may occur readily in this setting; infection rates of up to 60% have been reported in some series (93, 94, 43, 96, 97, 98, 99).

1.5.2 Sources of CMV
Primary infections may cause severe disease and in some cases may be fatal; latent virus may be acquired from donor tissue (43) and/or transfused blood (100).

Recipients that have previously encountered CMV also host active CMV infection after immunosuppression; however, such secondary infections generally cause less disease than primary infections (93, 94, 43, 96, 98, 99). Secondary infection may occur as a reactivation of recipient virus or reinfection with a different strain originating from the donated organ.

Sutherland et al 1992 (43) showed that, for 120 liver transplant recipients, 88% of primary infections (n=18) were symptomatic compared to only 55%
of reinfections and reactivations (n=77). In addition, symptomatic primary infections were more severe; manifestations included CMV hepatitis, gastrointestinal complications and thrombocytopenia. Eight per cent of transplants between CMV antibody negative donor and recipients were complicated post-transplant by CMV infection; this was presumed to be due to transmission from transfused blood. Other reports have confirmed the relatively low importance of CMV transmission by blood products in this setting (93, 98, 99). Furthermore, some CMV antibody negative individuals harbour CMV DNA in monocytes, which have been shown to be a site of latency (102); therefore, the 8% rate of transmission via blood products (43) may be an overestimate.

1.5.3 CMV Disease after Liver Transplantation
Symptomatic CMV infection after liver transplantation usually occurs in the first few months and may manifest as fever, malaise, arthralgias, leukopenia, thrombocytopenia, hepatitis, interstitial pneumonitis, enteritis, chorioretinitis and disseminated disease (99).

Often the only symptom evident is fever (99) which is also a symptom of acute rejection. Rejection is treated by increasing immunosuppression whereas CMV infection is treated by lowering immunosuppression and/or administration of anti-viral drugs. Therefore, specific diagnosis of active CMV infection is crucial (see section 1.7).

1.5.4 Targeting Antiviral Treatment After Liver Transplantation
A number of antiviral treatments are currently used including administration of acyclovir, ganciclovir, foscarnet and immune globulin. Acyclovir is a nucleoside analogue that is highly effective against HSV which encodes the enzyme (thymidine kinase) responsible for converting
acyclovir into its active form (acyclovir triphosphate) which is a potent inhibitor of HSV DNA polymerase activity (85); acyclovir is less effective against CMV (85) which does not encode thymidine kinase.

However, both the nucleoside analogue ganciclovir and the drug foscarnet (phosphonoformic acid) reduce the activity of CMV DNA polymerase (103, 380). The active, triphosphate form of ganciclovir is thought to be produced after the activity of a number of cellular kinases that are upregulated after CMV infection (85). True prophylactic administration of intravenous ganciclovir (5 mg/kg/day) is more effective than high-dose oral prophylaxis of acyclovir (800 mg/kg/day) and can prevent CMV disease in liver transplant recipients (104, 105). However, this regime is undesirable because ganciclovir is myelotoxic; in addition, intravenous administration of ganciclovir or foscarnet requires prolonged hospitalisation. Oral ganciclovir has recently become available and is likely to be effective in reducing the impact of CMV disease.

CMV antibody negative bone marrow (261) and liver (96) transplant recipients have been shown to be at less risk of CMV disease after prophylaxis with high titre CMV immune globulin; however, this preparation is costly, requires intravenous administration and is probably inferior to antivirals (Alexander GJM; personal communication).

Targetting of individuals at risk of developing CMV disease for preemptive ganciclovir treatment (given when asymptomatic CMV infection is detected) is an attractive alternative because patients at no risk are spared unnecessary treatment. The incidence of CMV disease is reduced when this policy is employed but not eliminated (106, 107). Elimination
may be possible if a highly sensitive laboratory test is used so that preemptive treatment can be given earlier (see section 1.7).

1.5.5 Liver Transplantation at Addenbrooke's NHS Trust

The success of liver transplantation, measured in terms of patient and graft survival, has improved since programmes were first initiated. The population of patients transplanted and the frequency and causes of graft and patient loss vary between centres. Therefore, initial diagnoses and survival statistics of the patients transplanted during the period of this study need to be stated in order to make worthwhile comparisons with previous reports from this and other centres.

During the period of study (the past 4 years), 235 patients have undergone liver transplantation at Addenbrooke's Hospital. These patients had a male: female ratio of 1.06 and a median age 38 (range 7-67).

1.5.5.1 Primary Disease

Liver transplantation was used to treat patients presenting with a variety of disorders as shown in figure 1.3.
Figure 1.3  Types and proportions of primary disease in 235 liver allograft recipients at Addenbrooke's NHS Trust
1.5.5.2 Patient and Graft Survival

The five-year actuarial survival rate for patients is now approaching 75%; currently, the re-transplantation rate is 9.8%.

1.5.5.3 Causes of Graft Loss

Fifty patients were re-transplanted over the past 4 years; the causes of these graft losses are distributed as shown in figure 1.4.

Figure 1.4 Causes of graft loss for the last 50 re-transplanted patients at Addenbrooke's NHS Trust
Chronic rejection was the largest single cause of graft loss among these patients (38%).

1.5.5.4 CMV Disease Among Liver Transplant Patients at Addenbrooke's NHS Trust

Over the past four years, 8.6% of liver transplant recipients at Addenbrooke's NHS Trust had CMV disease. This was clinically defined after observation of fever, malaise, arthralgias, leukopenia, thrombocytopenia, hepatitis, interstitial pneumonitis, enteritis, chorioretinitis or disseminated disease and the association with active CMV infection was confirmed by laboratory testing.

1.6 Bone Marrow Transplantation (BMT) and Cytomegalovirus (CMV)

1.6.1 Obliteration of Cellular Immunity Facilitates Active CMV Infection

Allogeneic bone marrow transplantation (BMT) and volunteer unrelated donor (VUD) BMT occur when bone marrow is transplanted from HLA identical siblings and HLA identical unrelated individuals respectively; both are used to treat leukaemia. Pre-transplant conditioning is required to provide 'marrow space' for donor stem cells, to eradicate the recipients cellular immunity and therefore avoid graft rejection and to eradicate marrow malignancy (108). This is achieved by a combination of cyclophosphamide, anti-thymocyte globulin (ATG), cyclosporin and/or total body irradiation (TBI). Cellular responses are completely ablated and subsequent CMV disease is common and often severe.
Meyers et al (109) found a 50% incidence of symptomatic infection CMV and a 10-30% incidence of CMV-mediated interstitial pneumonitis (of which 80% of cases proved fatal).

1.6.2 Sources of CMV
In the early period of bone marrow transplantation, severe and fatal CMV disease occurred after transmission of CMV by CMV antibody positive blood and blood products. More recently, CMV antibody negative blood products have been provided. This has affected the incidence of CMV infection of CMV antibody negative recipients. De Witte et al 1990 (110) followed this regime and found the incidence of active CMV infection to be 86% and 0% in CMV antibody positive and CMV antibody negative recipients respectively; 50% of those with active CMV infection experienced CMV disease. Similarly, Sullivan et al 1990 (111) found that only 2% of CMV antibody negative recipients are infected post-transplant.

CMV antibody positive recipients are therefore at a high risk of CMV infection and disease (in contrast, donor CMV antibody positivity confers the highest risk of subsequent active CMV infection and disease after liver transplantation). CMV can be transmitted with the transplanted marrow (112, 113) but it is believed that reconstitution of the donor immune system (which is primed for a response to CMV) mediates protection.

1.6.3 Timing
Active CMV infection, tested by blood and urine culture, appears during a relatively discrete time period; median times of initial detection vary between centres from day 35 (114) to day 59 (115).
1.6.4 CMV Disease after Bone Marrow Transplantation

CMV disease in this transplant population includes fever, athralgia, malaise, interstitial pneumonitis (see section 1.6.6.4), enteritis, hepatitis and graft suppression (thrombocytopenia and neutropenia) (116). The association between CMV and graft-versus-host disease is discussed in detail later (see section 1.6.6).

1.6.5 Targetting Antiviral Treatment After Bone Marrow Transplantation

A number of antiviral preparations are available (see section 1.5.4); ganciclovir is the most efficient inhibitor of CMV replication studied to date.

Survival among bone marrow transplant patients with CMV pneumonia treated with ganciclovir after the onset of clinical symptoms is low (10%) and relapses are common (108). True prophylactic administration of full doses of ganciclovir from the time of transplantation, to all allogeneic bone marrow transplant patients without regard to clinical symptoms or laboratory diagnosis of active CMV infection, can prevent the development of CMV pneumonitis (109). However, ganciclovir prophylaxis in this guise is undesirable because common adverse effects of ganciclovir include neutropenia and thrombocytopenia (119).

A number of reports suggest that a compromise position can be clinically advantageous; early, pre-emptive treatment with ganciclovir, before the development of clinical symptoms, improves survival and reduces CMV disease (120, 121). In addition, low doses of ganciclovir, used in this manner, are effective; this minimises hospitalisation and toxicity.
To exploit this approach an early diagnosis of active CMV infection, before the onset of clinical symptoms, is essential to allow early, low-dose ganciclovir prophylactic treatment to be targeted to individuals at high risk of developing CMV pneumonitis.

1.6.6 Graft Versus Host Disease (GVHD) after Allogeneic Bone Marrow Transplantation

Risk factors for the development of GVHD were listed by Billingham 1966 (122). The transfusion of immunocompetent cells from donor to recipient; T-lymphocytes are important and the number transfused is associated with disease severity (123). Antigenic differences between donor and recipient and recipient immunosuppression are also required.

Thus, a patient undergoing allogeneic bone marrow transplantation is at high risk of developing GVHD. A link has been proposed between cytomegalovirus infection and GVHD (see Chapter 3).

1.6.6.1 Acute GVHD

Acute GVHD after allogeneic bone marrow transplantation has an incidence of 10-80% and is responsible for severe morbidity and mortality (124). Damage to the epithelium of gut, liver and skin is initiated after T-lymphocyte infiltration (125). As the disease progresses the degree of inflammation increases and a complex array of cytokines are produced (126). Tumour necrosis factor α (TNF-α) production is important and mediates necrosis without the necessity of cell-cell interaction (127).

Risk factors for the development of acute GVHD include HLA mismatching, donor leukocyte infusion, recipient immunosuppression, sex mismatching and possibly donor herpesvirus serology (124).
1.6.6.2 Chronic GVHD

The incidence of chronic graft versus host disease (GVHD) after HLA-identical sibling transplantation is approximately 50% (128).

The mechanism of epithelial cell damage is similar to that of acute disease; epithelial cells are infiltrated by donor CD8+ T-lymphocytes. Cellular and humoral immunity are directed towards undefined recipient antigens and this is associated with severe immunodeficiency; this relationship has not been investigated fully but may occur because of exhaustion of factors required to mount T-lymphocyte activation. Infection in this setting is often severe and may be fatal (129).

Risk factors are preceding acute GVHD, recipient age and administration of donor buffy coat cells with marrow (129).

1.6.7 Allogeneic Bone Marrow Transplantation at Addenbrooke's NHS Trust: Data from the Past Five Years

Initial diagnoses and survival statistics of the patients transplanted during the period of this study need to be stated in order to make worthwhile comparisons with previous reports other centres.

Over the past five years, 58 bone marrow allografts have been performed at Addenbrooke's NHS Trust; 48 transplants were from HLA-identical siblings and 10 were from HLA-identical unrelated donors. This group comprised 28 male and 30 female recipients and had a median age 36.5 years (range 16-53 years).
1.6.7.1 Primary Disease.

BMT was used to treat patients with a variety of blood malignacies (see figure 1.5).

Figure 1.5 Types and proportions of primary disease in 58 bone marrow allograft recipients at Addenbrooke's NHS Trust.
1.6.7.2 Post-Transplant Mortality

A total of 41% (24/58) recipients have survived to date; mortality rates among sibling and unrelated-donor transplants are 50% (24/48) and 100% (10/10) respectively.

Causes of mortality are distributed as shown in figure 1.6.

Figure 1.6 Causes of death for 34 bone marrow transplant patients at Addenbrooke's NHS Trust
Infection accounted for 24% (8/34) deaths; CMV pneumonitis accounted for 2/8 cases of fatal infectious disease (see section 1.8.4). Chronic GVHD was responsible for 15% deaths (see section 1.8.4).

Cases of acute GVHD were not fatal but were associated with considerable morbidity (see section 1.8.4). Of the 58 patients allografted, 41% (24/58) experienced acute GVHD; moderate to severe (grade 3) and severe (grade 4) disease occurred in 19% (11/58) patients.

1.7 Diagnosis of Active CMV Infection

Pre-emptive ganciclovir treatment (see sections 1.5.4 and 1.6.5) requires a laboratory test of high specificity and sensitivity, with the ability to predict the subsequent development of CMV disease.

1.7.1 Conventional Tests

A number of methods are available for the diagnosis of CMV infection. Serological diagnosis depends on the appearance of specific IgM antibodies or a four fold increase in IgG titre (189). Such antibodies usually appear two to four weeks after the onset of CMV replication; serological diagnosis is, therefore, of limited use in relation to prompt antiviral treatment. Furthermore, antibody responses may be delayed in transplant patients due to immunosuppression. CMV may be isolated from buffy coat, urine and other specimens and cultured in human fibroblast cells. This method is regarded as the gold standard with respect to specificity and sensitivity but cytopathic changes occur too late to influence treatment. The detection of early antigen fluorescent foci (DEAFF) is a widely used detection method (190). The DEAFF test is performed by centrifugal inoculation of CMV from various clinical samples onto human fibroblasts, incubation
for 24 hours and then application of fluorescein labelled immediate early and/or early CMV monoclonal antibodies to the cells. This rapid test may be completed within 24-48 hours and its specificity and sensitivity are almost comparable with that of virus isolation. A problem encountered by both culture and the DEAFF test is that of reliability; a significant number of culture and DEAFF tests on blood and urine samples fail to give a result because of toxic effects of the specimen on the cells or microbial contamination.

1.7.2 Dot-Blot Hybridisation and Polymerase Chain Reaction (PCR)

1.7.2.1 Testing of Urine

Transplant patients with CMV infection usually excrete CMV in urine. Dot-blot hybridisation and the polymerase chain reaction (PCR) are potentially valuable tests in this field but major problems have been experienced with hybridisation specificity (157, 158, 159) and urine PCR inhibitors (160, 161, 162, 163, 164) respectively.

Kidd et al (1993) (160) reported testing of urine by PCR, culture and DEAFF in a mixed population of organ transplant recipients and patients with AIDS. PCR was most sensitive and detected active CMV infection earlier than culture or DEAFF (by 22 and 10 days respectively). PCR positivity was significantly associated with CMV disease.

1.7.2.2 Polymerase Chain Reaction (PCR) Testing of Blood

A number of reports suggest that blood PCR positivity has a better correlation with CMV disease than urine positivity (160) after liver transplantation. Furthermore, PCR testing of blood has been shown to be

1.7.2.3 PCR Positivity and Disease

PCR positivity does not necessarily correlate with symptomatic infection. Gerna 1991 (166) reported that, for 14 heart transplant recipients, blood PCR positivity in the absence of positive DEAFF or isolation results was not associated with CMV disease.

However, quantitation of CMV DNA levels may determine a threshold above which symptomatic infection is likely. Gerna 1991 (166) found such a threshold of viraemia in 14 heart transplant recipients using conventional cell culture.

Attempts have been made to measure CMV load in clinical samples using semi-quantitative and quantitative PCR (180, 181, 182, 183). The latter is typically performed by amplifying exogenous controls, of known quantity, alongside samples or by limiting dilution.

1.8 Chronic Rejection after Liver Transplantation: A role for CMV?

1.8.1 Histopathology of Liver Allograft Rejection

1.8.1.1 Acute Rejection

The incidence of acute rejection in liver allograft recipients is 70% (191).

Snover et al 1984 (192) defined the following criteria for the diagnosis of acute rejection based on portal tract histology:
1. Inflammatory infiltration of portal tract involving lymphocytes, monocytes, plasma cells, neutrophils and eosinophils.

2. Inflammatory damage to small and medium sized bile ducts involving mononuclear and polymorphonuclear cells; damage to the biliary epithelium may occur.

3. Venous endothelial inflammation involving subendothelial inflammation in severe cases.

Parenchymal lesions may also occur and include lymphocytic infiltration, cholestasis, hepatocyte ballooning and hepatocyte necrosis. Parenchymal involvement is caused by direct immune mediated injury or ischaemia (191, 193).

Histological studies suggest that the sites of initial damage are the bile duct endothelium and epithelium (193). Inflammatory infiltrates predominantly consist of T-lymphocytes; CD4+ cells predominate before clonal expansion of the CD8+ fraction leads to a high ratio CD8+ : CD4+ (191, 193).

1.8.1.2 Chronic Rejection

The incidence of chronic rejection after liver transplantation varies between 5-20%. In most cases the initial time of onset is 3-6 months.

Hubscher 1991 (191) defined two criteria for the diagnosis of chronic rejection;

1. Vanishing bile duct syndrome (VBDS)
2. Obliterative vasculopathy of small and medium sized arteries

VBDS is a progressive disorder which cannot be treated and leads to graft loss. Initial inflammation of portal tracts, associated with bile duct damage, is histologically similar to that seen in acute rejection. However, initial inflammation may be unnoticed. Infiltrates comprise predominantly T-lymphocytes plus natural killer cells and neutrophils. Portal tract inflammation precedes progressive loss of bile ducts, which is not a feature of acute rejection (191, 193).

Arteries are also involved; endothelial damage leads to infiltration of the intima by foamy macrophages and occlusion of the lumen. Subsequent ischaemia, together with direct immune damage, leads to hepatocyte necrosis and interstitial fibrosis (191, 193). In some cases, vascular disease precedes VBDS.

1.8.2 Risk Factors for Chronic Rejection after Liver Transplantation

1.8.2.1 A Comprehensive Study

To date, the most comprehensive study was performed by Cardinas et al (1995) (210); 423 consecutive primary liver transplants were analysed (chronic rejection incidence 5.2%). Risk factors were pre-transplant diagnosis of primary biliary cirrhosis (PBC) (relative risk 10.6; p=0.006) or autoimmune hepatitis (relative risk 6.7; p<0.05), recipient age < 30 years (relative risk 3.8; p=0.015), acute rejection episode(s) (relative risk 3.6; p=0.03) and transplantation from a male donor into a female recipient (relative risk 3.03; p=0.04). CMV antibody mismatch (donor CMV antibody positive : recipient CMV antibody negative) was also positively associated
with chronic rejection (relative risk 3.5; p=0.015). In contrast to other reports (see below), human leukocyte antigen (HLA) mismatch at the A, B or DR loci, diagnosis of primary sclerosing cholangitis pretransplant and symptomatic CMV infection were not associated with chronic rejection.

1.8.2.2 The Effect of HLA Matching upon Chronic rejection: Suggested Links with CMV and Autoimmune disease.

Reports of an association between HLA matching/mismatching and chronic rejection have been conflicting.

A study by the Kings College group (243) investigated 62 liver transplant recipients, 14 (23%) of whom developed chronic rejection. HLA DR matching of 1 or 2 loci (relative risk 2.5; p<0.025) and zero HLA A,B matching (6.2; p<0.05) were independently associated with the incidence of chronic rejection. The authors suggest that matched DR HLA present foreign A,B HLA peptides and facilitate MHC-restricted interaction and subsequent immune-mediated damage.

This group confirmed these findings in a second study (224) of 101 liver transplant patients (17% chronic rejection). Chronic rejection was positively associated with HLA DR matching (relative risk 9.4; p<0.002) and HLA A and B mismatching (3.1; p<0.05). In addition, chronic rejection was associated with active CMV infection (7.5; p<0.005). A 1-2 HLA DR match combined with CMV disease comprised the highest relative risk for rejection suggesting synergy. The authors suggest that MHC class II restricted presentation of foreign HLA A,B and viral peptides leads to chronic rejection.
Interestingly, the recent report from the Birmingham group (210) did not find such a correlation between active CMV infection and chronic rejection. It is possible that the introduction of ganciclovir, subsequent to the Kings College report, is responsible for this conflict; 33% and 8% of patients suffered symptomatic CMV infection respectively in these two studies.

The findings of the Kings College group differ with those of the Pittsburgh group. Markus et al (1988) (240) studied 507 liver allograft recipients and found that, although HLA-DR matching was associated with reduced graft survival (p=0.054), mismatching of this locus was associated with chronic rejection (p=0.007). These authors suggest that DR matching facilitates the recurrence of autoimmune diseases such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and alcoholic cirrhosis.

Subsequently, the Pittsburgh group (225) studied a series of 399 liver transplants having a 7% incidence of CMV hepatitis and an 8% incidence of chronic rejection. The incidence of chronic rejection was associated positively with the incidence of CMV hepatitis (24% versus 6%; p=0.0007). In addition, chronic rejection occurred earlier in the CMV hepatitis positive group (median 60 versus 245 days; p=0.07). Furthermore, the incidence of CMV hepatitis was higher in HLA-DR matched transplants in both CMV antibody negative (44% versus 14%; p=0.07) and CMV antibody positive (12% versus 2%; p=0.006) recipients. However, DR matching per se was not associated with the incidence of chronic rejection. The authors propose that the immunopathology of CMV hepatitis is mediated by MHC-restricted presentation of CMV peptides and is associated with the development of chronic rejection.
It is difficult to consolidate these reports. The Birmingham group (210) found no relationship between HLA mismatching and the incidence of chronic rejection but failed to score for HLA matches; therefore, this paper cannot be compared directly with the Kings College papers (243, 224). Functional relevance is important when scoring matches or mismatches e.g. HLA molecules with antigenic dissimilarity may be able to present similar peptides if peptide binding grooves are identical.

The effects of HLA upon chronic rejection may be 'dualistic'. For one HLA allele it is not inconceivable that matching for MHC-restricted presentation and mismatching for antigenic variability can both be associated positively with chronic rejection.

Such dualism of a single HLA allele may have been demonstrated by the Pittsburgh group (see above). Here, DR matching is associated with CMV hepatitis and graft loss but not with chronic rejection. DR mismatching and CMV hepatitis are associated with chronic rejection. If more transplants were studied then a direct link between DR matching and chronic rejection may emerge.

1.8.2.3 The Importance of Tissue Invasion by CMV

1.8.2.3.1 Persistent CMV Infection in Chronically Rejected Liver Allografts

This question was addressed by the Kings College group after describing the link between active CMV infection and chronic rejection. Arnold et al 1992 (284) used in situ hybridisation to screen serial liver biopsies taken from patients with VBDS or uncomplicated CMV hepatitis. CMV DNA was found in the hepatocytes of all patients with CMV hepatitis and 10/12
of those with VBDS. The latter group were persistently CMV positive until the graft was lost whereas the CMV hepatitis group cleared CMV from hepatocytes \((p<0.0005)\). CMV was not found in the principal sites of damage (see section 1.8.1) i.e. bile ducts and arterioles. However, CMV infection of hepatocytes may indirectly cause or promote damage of these tissues, for example by mediating clonal expansion of allospecific T-lymphocytes (see section 1.9.3) or by affecting lymphokine and/or adhesion molecule levels (see section 1.9.4). An actively infected, immunostained, bi-nucleate hepatocyte is shown in figure 1.7 (see Chapter 6).

**Figure 1.7** A Hepatocyte Permissive to Active CMV Infection Identified by Immunohistochemistry (original magnification x200; arrow indicates positive cell).

CMV infection of liver graft endothelial or epithelial cells has not been previously reported. However, Patterson et al (1988) (381) described development of necrotic skin lesions in a liver transplant recipient;
vascular endothelial cells and epithelial cells were cytomegallic and active CMV infection was confirmed by in situ hybridisation (see also Chapter 6).

1.8.2.3.2 Endothelial and Epithelial Cells are Permissive to Active CMV Infection In vitro

CMV infects 5-10% of endothelial cells in vitro (249); these cells have been implicated in chronic renal, heart and liver rejection (see section 1.8.3.2).

CMV also infects 5% cultured tubular epithelial cells (217, 250) which are involved in acute renal rejection. Epithelial cells are permissive to CMV replication (250).

Chronic rejection-mediated damage is associated with fibrosis. It has been shown in vitro that fibroblasts are permissive to CMV infection and replication (282).

1.8.2.4 Are Antibodies a Risk Factor for Rejection of Liver Allografts?

Liver grafts are considered to be relatively refractory to the presence of alloreactive lymphocytotoxic antibodies pretransplant (289, 290, 291, 292, 293) and posttransplant (287) when compared to studies of renal transplant recipients (see sections 1.8.3.3.2 and 1.8.3.3.3). Gordon et al (295) presented data from 337 primary liver transplants; the incidence of retransplantation after rejection was similar for the 'positive crossmatch' group (n=38) and controls (11.3% versus 12.4%).
However, other groups have presented data suggesting a link (293, 294, 286). Batts et al (1988) (293) used a more sensitive detection technique (antihuman globulin-complement dependent cytotoxicity assay (AHG-CDC)) and found a higher incidence of crossmatching (31%; 16/52). Chronic rejection was associated with pretransplant crossmatch positivity (83% (5/6) versus 24% (11/46); p<0.004).

In another study (294), the authors speculated that the previously observed insignificance of crossmatching occurred because IgM antibody (detected in conventional CDC) did not play a role in chronic rejection. Therefore, IgM was eliminated in this study after treating serum samples with dithiothreitol. Significantly higher incidences of graft failure and cellular rejection (chronic and acute) were found in a group of IgG crossmatch positive patients (n=26) compared to controls (n=52). However, crossmatching did not influence the incidence of chronic rejection per se.

1.8.2.5 Are Primary Autoimmune Disorders a Risk for Chronic Rejection?

Most centres report successful liver transplantation after primary sclerosing cholangitis (PSC) (194, 195, 196), primary biliary cirrhosis (PBC) (197, 198, 199) and autoimmune hepatitis (AIH). Circulating antibodies have been associated with these autoimmune hepatic disorders (see section 1.9.6); successful outcome after transplantation suggests that other factors are important.

However, recurrence of PBC (200, 201, 202), PSC (203) and AIH (204) may occur after transplantation. Some reports contend that PBC (197, 210) and PSC (203, 205, 206) are linked with liver allograft chronic rejection.
1.8.3 Other Allografts

1.8.3.1 HLA Matching Benefits Renal and Cardiac Transplantation

The benefits of HLA matching upon outcome have been documented for renal and cardiac transplantation (232, 233, 234, 235, 207).

Morris et al (1993) (207) studied 148 renal transplant recipients whose grafts were optimally matched for HLA-A, -B, and -DR. Mismatching was significantly associated with an increased incidence of acute rejection episodes (p<0.005) and reduced graft survival (p<0.03).

1.8.3.2 CMV is a Risk Factor for Rejection After Renal and Cardiac Transplantation

Data from a number of papers suggests a link between CMV infection and rejection of renal (207, 211, 214, 217, 218, 219), cardiac (228, 222), lung (209) and intestinal (208) allografts.

The findings of Von Willebrand (1986) (214) are compelling; in a series of 237 renal allograft transplants the incidence of CMV disease, diagnosed by culture and clinical symptoms, was 6%. The incidence of rejection was significantly higher in patients with CMV disease (86% versus 17%; p<0.001) and rejection episodes were concomitant with the diagnosis of CMV disease.

The largest study of cardiac allografts was performed by Gratton et al (1989) (228, 229). In 389 heart transplant recipients the incidence of active CMV infection was 30% (91/301). Upon comparison of those with active CMV infection with those with no evidence of active CMV infection it was shown that the former group had a significantly higher incidence of acute rejection episodes (p<0.001).
rejection, rate of acute rejection (episodes/100 days post transplant), death rate from atherosclerosis at five years post-transplant and correspondingly, a significantly lower overall survival rate at five years post-transplant.

1.8.3.3 CMV Infection of Graft Tissue After Renal or Cardiac Transplantation

The endothelium provides a barrier between the graft and the recipient's immune system. CMV infection of endothelial cells may be an important initiator or modulator of graft rejection; CMV has been shown to be tropic for renal and cardiac graft endothelial cells (382, 383, 384, 385, 386). In addition, it has recently been shown that murine endothelial cells harbour latent murine CMV (44).

Renal Grafts

Payton D et al (1987) (383) described a renal transplant recipient with CMV infection (observed after light microscopy) of glomerular and peritubular endothelial cells and tubular epithelial cells. Active CMV infection of dermal vascular endothelial cells has been described for one renal transplant recipient (382). Furthermore, actively infected, circulating endothelial cells have been described after renal transplantation (387); the authors suggest that CMV uses these cells as a vehicle for dissemination.

Cardiac Grafts

Atherosclerosis is a vascular disease that has been linked to CMV infection and is accelerated after heart transplantation (388). This association is of particular importance because chronic rejection of liver grafts is often initiated by hepatic artery occlusion (191, 193; see figure 1.8) and histology is similar to that seen for atherosclerotic coronary arteries (figure 1.9 (Alexander GJM; unpublished observations)).
Occlusion of the lumen after intimal thickening is characteristic of both atherosclerotic untransplanted coronary arteries and chronically rejected, transplanted hepatic arteries (see figures 1.8 and 1.9 overleaf). Furthermore, intimal thickening involves proliferation of macrophages in both diseases; the processes leading to proliferation of these cells may be similar.

Hendrix et al (1989) (389) detected CMV by PCR in femoral or abdominal arterial samples from non-transplanted, atherosclerotic patients and normal controls; a greater proportion of atherosclerotic patients tested positive (90% (40/44) versus 53% (18/34)). Furthermore, it has been shown that a significantly higher proportion of non-transplanted, atherosclerotic patients were CMV antibody positive compared to controls (382).

CMV DNA has been detected in endothelial cells, lymphocytes and smooth muscle cells of coronary arteries after cardiac transplantation; 6/19 grafts were positive after in situ hybridisation (385).

Herpesvirus involvement with atherosclerosis has been proven in a chicken model. Fabricant et al (1983) (392) found that chickens developed atherosclerosis after infection with Mareks disease virus (MDV) and that disease was accelerated after feeding with cholesterol; uninfected animals did not develop atherosclerosis.

In a review article, Melnick et al (1995) (388) hypothesises that atherosclerosis is initiated after CMV-mediated endothelial damage. Exposed smooth muscle cells are damaged and subsequently proliferate; this leads to intimal thickening and lumen occlusion.
Figure 1.8 Occlusion of a Hepatic Artery Due to Chronic Rejection

Figure 1.8 shows a cross section of a medium sized hepatic artery from a liver graft that was explanted after chronic rejection. A subintimal aggregation of foam cells (macrophages) led to complete occlusion of the lumen. Intima (I), foam cells (F). Magnification x 200.

Figure 1.9 Atherosclerotic Coronary Artery

Figure 1.9 shows an untransplanted, atherosclerotic coronary artery (proximal, right). The intima (I) is thickened by an atherosclerotic plaque (A) containing macrophages, calcium and lipid which is capped by a proliferation of smooth muscle cells (SM). The lumen (L) is occluded. Magnification x 400. Photograph kindly provided by Professor Peter Weissberg.
1.8.3.4 Alloreactive and Autoreactive Antibodies and Rejection after Renal and Cardiac Transplantation

1.8.3.4.1 Preformed Alloreactive Lymphocytotoxic Antibodies and Hyperacute Rejection

The presence of preformed alloreactive lymphocytotoxic antibodies in renal graft recipients has been shown to elicit hyperacute rejection (296, 297, 298) which occurs within minutes after restoration of blood flow. Such antibodies are alloreactive and are directed against human leukocyte antigens (HLA). Cells expressing HLA in vivo are targeted; graft cells have been shown to express class I and, to a lesser extent, class II MHC molecules (see section 1.9).

Patel and Terasaki 1969 (297) published the results of a multi-centre study of 248 renal transplant recipients. Pre-transplant serum from each recipient was assayed for antibody against a panel of lymphocytes from 10-40 individuals as well as graft donor lymphocytes. The presence of preformed lymphocytotoxic antibodies was associated with hyperacute rejection (43% (34/80 grafts versus 2% (4/168); p=0.001); this correlation was stronger when donor specific antibodies were present i.e. a positive crossmatch was seen (80% (24/30 grafts) versus 4.1% (8/195); p<0.001).

1.8.3.4.2 Alloreactive Lymphocytotoxic Antibodies Post-transplant

The development of alloreactive lymphocytotoxic antibodies post-transplant is also associated unfavourably with outcome (300, 301, 302, 303, 304, 305). Halloran et al 1992 (302) tested serially taken serum samples from 64 renal transplant recipients against a panel of lymphocytes. The development of lymphocytotoxic antibodies was associated with an
increased incidence of rejection (100% (13/13) versus 41% (21/51); p<0.005) and graft loss (38% (5/13) versus 4% (2/51); p<0.002). The authors suggest that rejection of renal grafts may be mediated by cellular or antibody responses and contrast the histopathology of each.

Antibody production during rejection episodes was thought to be artefactual by McCarty et al 1984 (306) after screening 42 renal transplant recipients for post-transplant production of auto-antibodies and lymphocytotoxic antibodies. Antibody responses were associated with graft loss but not with the occurrence of rejection episodes; the authors proposed that antibodies were formed after rejection-mediated damage and subsequent exposure of immunogens.

1.8.3.4.3 Non-Lymphocytotoxic Antibodies

Renal transplantation

A number of reports have documented the association between graft rejection and non-lymphocytotoxic antibodies (305, 306, 307, 308, 309, 310, 311).

Soulillo et al 1981 (305) investigated the specificities of immunoglobulins deposited in rejected renal allografts. Immunoglobulin was only found in grafts removed after severe acute rejection (62% IgG positive) or chronic rejection (75% IgG positive). A proportion of eluted antibodies were lymphocytotoxic. Immunohistochemical application of eluates to normal kidney sections showed that glomerular and tubular basement membranes and DNA were also targets.
Similarly, McPhaul et al 1981 (307) found that 35 rejected renal grafts contained antibodies directed specifically to renal structures. In addition, antibodies bound to smooth muscle and endothelium cells. Some anti-smooth muscle antibodies were also lymphocytotoxic.

Other reports also document a correlation between poor graft function and the occurrence of anti-endothelial cell antibodies pre- (312) and post-transplant (308).

Cardiac Transplantation
The effect of pre-transplant antibodies upon the outcome of cardiac grafts was investigated by Latif et al 1995 (309). Pre- and post-transplant sera from 129 heart transplant recipients was screened by polyacrylamide gel electrophoresis (PAGE) and Western blotting against normal heart tissue. Low amounts of anti-heart IgG and IgM and were found in 48% and 45% of recipients respectively. In addition, 22.5% of recipients harboured large amounts of anti-heart IgM and these recipients experienced more rejection episodes (p=0.001). A number of immunogens were characterised; tropomysin (35kD), actin (42kD), heat shock protein-60 (HSP-60; 60kD), HSP-70 (70kD) and myosin (200kD).

After injection of LEW rats with DA rat heart homogenate a mixture of auto- and allo-antibodies were induced (311). Auto-antibodies were directed against heart-specific targets whereas allo-antibodies, studied after pre-absorbtion with LEW heart homogenate, reacted specifically with donor spleen cells (presumably against MHC antigens) and donor heart antigens.
1.8.4 Does CMV have a Causal Relationship with Rejection?

Although significant associations between rejection events and CMV antibody status may be found it is difficult to establish whether CMV has a causal relationship with rejection.

A number of authors have hypothesised that active CMV infection occurs as a result of further immunosuppressive treatment for rejection episodes (219, 220, 221, 222, 234). Burd et al (1994) (219) found, in 135 paediatric renal transplant recipients, that treatment for acute rejection was a risk factor for the development of CMV disease.

In contrast, Pouteil-Noble et al (1993) (218) have elegantly shown that active CMV infection can be a risk factor for subsequent rejection. Of 242 renal transplant recipients, 157 patients (65%) developed active CMV infection whereas 85 patients (35%) remained CMV free. In a retrospective analysis, each CMV-free recipient was paired with a CMV infected individual to create 85 pairs. For each pair, the occurrence of rejection episodes after the time of diagnosis of CMV infection (in the CMV infected individual) was noted for both individuals. Rejection after CMV diagnosis occurred in 45% (38/85) of CMV infected individuals and 10.6% (9/85) of CMV free individuals (p<0.0001).

Ackermann 1988 (212) published the results of a policy to graft CMV antibody negative recipients only with kidneys from CMV negative donors; graft loss (0% versus 2.5%; p=0.016) and mortality (0% versus 3.7%; p=0.002) were reduced compared to historical controls. This is evidence for the hypothesis that CMV initiates or enhances the rejection process in renal grafts.
1.9 Mechanisms of Rejection

1.9.1 HLA Class II Matching May Facilitate Presentation of Foreign MHC or CMV Peptides

1.9.1.1 Hypothesis
Some authors have hypothesised that HLA matching, in the context of liver transplantation, plays a dualistic role.

Markus (1988) (240) suggest that DR matching facilitates the recurrence of autoimmune diseases such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and alcoholic cirrhosis.

Other authors propose that HLA matching eliminates non-MHC restricted cellular immune injury (242) but facilitates MHC restricted presentation of graft antigens. MHC restricted presentation of Y chromosome specific (210), mismatched HLA class I (224, 243) or CMV antigens (224) may occur (see section 1.8.2).

1.9.1.2 Evidence
There is evidence to support the hypothesis that matching facilitates MHC-restricted presentation of graft antigens. Fung et al 1986 (244) and Markus et al (1987) (245) have both demonstrated that lymphocytes directed against specific HLA antigens infiltrate liver grafts during rejection episodes.

MHC-restricted lymphocytes directed against specific CMV antigens have been documented (Quinnan et al 1981 (246); class I restriction, Lindsay et al
1986 (68) and Gehrz et al 1987 (248); class II restriction); infiltration of such cells during rejection episodes has not been shown.

In contrast, Waldman et al (1992) (262) suggested that CMV protein IE 2 may be able to present peptides to T-lymphocytes. T-cell proliferation in the presence of allogeneic endothelial cells was demonstrated. Proliferation of CD4+ T-lymphocytes (CMV antibody positive donor) was significantly enhanced when target cells were CMV infected. MHC-restricted presentation of CMV antigens was impossible because target cells were allogeneic; the authors speculated that sequence homology between CMV IE-2 protein and the HLA DR β chain, reported by Fujinami et al (1988) (263) may be important.

1.9.2 CMV and HLA Regulation

1.9.2.1 Hypothesis
CMV infection regulates expression of graft HLA which in turn modulates rejection processes.

1.9.2.2 Evidence

CMV Upregulates HLA Class I Expression in Epithelial, Endothelial and Smooth Muscle Cells.
The effect of CMV upon epithelial and endothelial cells is of great importance because these cells are the sites of initial rejection-mediated damage (see sections 1.8.1 and 1.8.2.3.2).

CMV infection has been shown to increase the surface levels of class I MHC molecules. Van Dorp 1993 (249) and 1989 (250) used a radioimmunoassay to demonstrate augmentation of HLA class I surface
levels for proximal tubular epithelial cells and human umbilical endothelial (HUVE) cells *in vitro*; 5% and 10% of these cells became infected respectively and class I expression initially increased 24 hours post-infection, peaking 48-72 hours post-infection. CMV infection had no effect upon surface expression of HLA class II but did not render cells refractory to class II upregulation by interferon (IFN)-γ. The upregulation of class I expression was not cytokine mediated; supernatants from CMV infected cell cultures had no effect on class I or II expression therefore excluding the effect of virus induced cytokines.

In contrast, Hosepud 1991 (251) was unable to show HLA upregulation in CMV infected HUVE or human aortic endothelial cells (infectivity 10%) but did show class I upregulation in human aortic smooth muscle cells (infectivity 100%). Flow cytometry was performed after application of monoclonal antibodies to HLA class I or II; it is possible that this assay was not as sensitive as the radioimmunoassay used by Van Dorp et al 1989 (249) and 1993 (250).

**CMV Downregulates HLA Class I Expression in Fibroblasts**

It is possible that the effect of CMV infection upon HLA expression is cell-type specific; other workers have demonstrated CMV-mediated HLA class I downregulation in fibroblasts. Del Val et al 1992 (252) and Beersma et al 1993 (253), working with murine and human CMV respectively, show that a CMV early gene is responsible for blocking HLA class I transport from the endoplasmic reticulum and thus reducing surface expression. Class II expression was unaltered and was induced by IFN-γ.
HLA Surface Expression in vivo During Active CMV Infection

Von Willebrand et al 1986 (214) (see section 1.8.3.2) suggested that CMV upregulation of HLA class II is an important factor during renal graft rejection. Immunohistochemistry for class II HLA molecules showed that the expression of HLA class II upon endothelial and tubular cells occurred subsequent to active CMV infection.

Several reports of HLA class II upregulation in rats infected with rat CMV support the findings of Von Willebrand et al 1986 (214) (Ustinov et al 1994 a (254) and b (255), Lemstrom et al 1995 (256). Ustinov et al 1994 a (254) and b (255) studied the effects of rat CMV in untransplanted animals thus eliminating the possible effects of rejection. HLA class II upregulation was seen in cells which are targets for immune mediated damage during rejection i.e. kidney vascular endothelium, tubular cells and glomeruli, liver vascular endothelium and parenchyma and heart capillary endothelium.

1.9.3 Alloreactive Cellular Immune Responses may be Augmented by CMV

Grundy and Shearer 1984 (257) describe the effect of primary infection of murine CMV (Smith strain) upon alloreactive T-lymphocytes in non-transplanted mice. Initially, the response was diminished following infection (days 2-4) but rose and was greatly enhanced from days 7-13.

Bulk cultures of lymphocytes from endomyocardial biopsy specimens, taken from heart transplant recipients, were tested for alloreactivity by Ouwehand et al (1994) (258). A significantly greater proportion of biopsy-derived cultures taken from patients with active CMV infection, had alloreactive activity (88% (74/84) versus 74% (173/234); p=0.01). The
authors suggest that HLA molecule and/or adhesion molecule (see sections 1.9.2 and 1.9.4) upregulation by CMV caused this phenomenon. Other possibilities exist, e.g. IFN-γ production by CD4+ cells directed against MHC-restricted CMV targets could cause clonal expansion of CD8+ cells to MHC-restricted or -unrestricted allogeneic targets.

1.9.4 CMV Upregulates Inflammatory Markers

CMV disease is associated with enhanced levels of lymphokines in serum and tissue. Elevated serum levels of IL-2 (264), IL-6 (370, 434), IL-8 (371, 433) and TNF-α (399) have been documented during episodes of active CMV infection after transplantation.

Cell surface expression of adhesion molecules stabilizes interactions between T-lymphocytes and target cells. CMV disease after liver transplantation is associated with enhanced surface expression of ICAM-1 on hepatocytes (369). Similarly, elevated surface expression of VCAM-1 and ICAM-1 on hepatic vascular and sinusoidal endothelium has been associated with active CMV infection after liver transplantation (435). Craigen and Grundy 1996 (436) showed that CMV upregulated ICAM-1 and LFA-3 levels in vitro using fibroblasts; this is an important result because it could be argued that observations in vivo are due to early rejection events.

The expression of lymphokines and adhesion molecules is enhanced during rejection and therefore these markers cannot be used to discriminate between CMV disease and rejection.
Upregulation of adhesion molecule and lymphokine expression by CMV may initiate or increase the severity of cellular immune-mediated damage after transplantation.

1.9.4.1 Tumour Necrosis Factor-α (TNF-α), CMV and Chronic Rejection of Liver Grafts

Tumour necrosis factor-α (TNF) is a cytokine that was originally described by Carswell et al (1975) (393). Here, production of murine TNF was stimulated by injection of bacterial endotoxin and led to haemorrhagic necrosis of transplanted subcutaneous tumours; production of TNF was predominantly by macrophages (394). In addition, TNF-α has been linked with acute (395, 396) and chronic (397) rejection of liver grafts.

Moreover, there is evidence to suggest that active CMV infection is induced by TNF (398, 399). Docke et al (1994) (398) have shown that patients with septic disease are more likely to have detectable levels of TNF in plasma by immunoassay (61% (27/44) versus 0% (0/44)) and active CMV infection by PCR of peripheral blood mononuclear cells (PBMC) (92% (23/25) versus 7% (3/44); p<0.001).

Conversely, active CMV infection may upregulate TNF production. Geist et al (1994) (402) found that the CMV proteins IE 1 and IE 2 upregulated the TNF promoter (using a CAT reporter construct transfected into the monocytic cell line THP-1). This effect was also apparent after active CMV infection of differentiated THP-1 cells.

The quantity of TNF produced by an individual is partly governed by the genetics of two TNF promoter alleles. The TNF-2 promoter allele differs from TNF-1 by a single base pair mutation in the promoter region of this
gene (guanine (G) and alanine (A) residues are positioned at 308 bases upstream of the transcription start site respectively). *In vitro* studies using a CAT reporter construct have shown that the TNF-2 promoter elicits higher basal and inducible expression compared to the TNF-1 promoter (405).

McGuire et al (1994) (403) analysed the frequencies of TNF-1 and TNF-2 alleles in 918 patients with malaria (475 cerebral malaria, 443 malarial anaemia) and 325 controls. The proportion of individuals with the TNF-2 allele was significantly higher for the cerebral malaria group (compared to malarial anaemic patients and controls) and was significantly elevated within the former group in those with severe or fatal cerebral malaria. TNF-2 was present in this Gambian population at a frequency of 0.16 and the authors suggest heterozygote advantage i.e. heterozygotes produce an optimal amount of TNF which may be sufficient to reduce the incidence of infection but insufficient to elicit cerebral malaria.

1.9.5 CMV Infection Modulates Autoantibody Production

1.9.5.1 CMV and Autoantibody Production

Kantor et al 1970 (312) performed cardiopulmonary bypass upon 39 patients. Ten developed active CMV infection; serum was collected and screened immunohistochemically against fixed peripheral blood lymphocytes. CMV infection was associated with the production of autoantibodies with activity against nuclear and erythrocyte antigens. One patient developed glomerulitis and it is suggested that CMV induces autoantibodies with specificities towards renal antigens.
This contention was supported by Andersen and Andersen 1974 (313). Three groups were tested for auto-antibodies; healthy CMV antibody negative individuals (n=40), healthy CMV antibody positive individuals and those with active CMV infection (n=9). A greater prevalence of smooth muscle antigen- (16% (10/63) versus 0% (0/40); p=0.005), nuclear antigen- (14% (9/63) versus 2.5% (1/40; p=0.046) and glomerular antigen- (3% (2/63) versus 0% (0/40)) specific auto-antibodies were found in CMV antibody positive individuals; 58% (5/9) and 44% (4/9) of those with active CMV infection had smooth muscle antigen- and glomerular antigen-specific auto-antibodies respectively. The importance of these autoantibodies after renal transplantation has been discussed (see section 1.8.3.3.3).

It has been shown *in vitro* that CMV infection of fibroblasts increases the cell surface expression of calreticulin (314) which is an autoantigen.

In addition, upregulation of class I surface expression by CMV infection may mediate or enhance anti-HLA antibody production (see section 1.9.2).

### 1.9.5.2 CMV and Autoantibody Production After Transplantation

Middleton et al 1981 (310) has shown that anti-smooth muscle antigen IgM is found in significantly more patients after renal transplantation (76% (53/70)) compared to pre-transplant patients on haemodialysis (14% (10/70)) and normal controls (5% (2/40)). A fourfold or greater increase in titre was seen in patients experiencing rejection and/or CMV infection and the authors propose a causal link.

Evidence may also be found in a murine model set up by Grundy and Shearer 1984 (257). Here, mCMV enhanced alloreactivity was described
(see sections 1.6.6.3, 1.6.6.4 and 1.9.3) and this coincided with an increase in auto-antibody production.

1.9.5.3 Cross-Reactive Antibodies

Production of antibodies cross-reactive between HLA class I and the CMV H301 gene product has been demonstrated (60). The authors postulate that CMV infection could increase production of antibodies directed to graft HLA motifs thus modulating the rejection process.

1.9.6 Rejection may be Modulated by Autoantibodies Associated with Primary Sclerosing Cholangitis (PSC), Primary Biliary Cirrhosis (PBC) and Autoimmune Hepatitis (AIH).

Some authors suggest that recurrence of autoimmunity following liver transplant can initiate or promote the rejection process (see section 1.8.2.5). The autoantigens associated with PSC, PBC and AIH are summarised below; this is integral to the Western blot results shown in Chapter 4.

1.9.6.1 Primary Sclerosing Cholangitis (PSC)

PSC is associated with a number of autoantibodies that act as markers (316, 317, 318). Snook et al (1989) (316) used immunohistochemistry to find that 84% of PSC patients harboured antibodies with specificity to neutrophil nuclei.

Klein et al (1993) (317) tested serum for anti-neutrophil nuclear antibody (ANCA) after Western blotting of sonicated neutrophils. Sera from PSC patients reacted with a number of antigens with molecular weights of 95, 60, 55, 40, and 30 kD. The idiotype is almost exclusively IgG (319). Eighty per cent of PSC patients were scored positive using this method compared to 9% ulcerative colitis, 10% Crohns disease, 11% PBC, 13% AIH and 0%
normal controls. None of the 24 PSC patients contained antibodies to all 5 antigens; 50% patients only had antibodies to the 60kD antigen.

A similar Western blotting protocol was used by Lo et al (1993) (318) who identified 6 'PSC antigens' of 205, 116, 97, 68, 45 and 29 kD. However, 50% patients without PSC and 50% normal controls harboured antibodies to these neutrophil antigens. The sensitivity of this test may be responsible for this discrepancy. This result is important; it suggests that healthy individuals harbour low titres of the autoantibodies involved in PSC.

**1.9.6.2 Primary Biliary Cirrhosis (PBC)**

PBC is characterised by circulating autoantibodies with specificity to mitochondrial proteins. Van de Water et al (1989) (320) found that serum from 93 patients with PBC identified three proteins when applied to Western blots of mitochondrial protein. These proteins are 74, 52 and 39 kD in size. The 74 and 52 kD proteins are pyruvate dehydrogenase complex-E2 component and branched-chain a-keto acid dehydrogenase complex-E2 component respectively; the 39kD protein may be a breakdown product of the 74kD protein. Sera from 85% patients with PBC reacted with the 74kD protein, 53% with the 52kD protein and 47% reacted with both. Sera from normal controls (n=86) and patients with PSC (n=38) did not react.

**1.9.6.3 Autoimmune Hepatitis (AIH)**

Stechemesser et al (1993) (321) purified liver tissue for Western blotting; 63% (111/175) sera from patients with AIH identified the 'liver-pancreas' antigen which manifests as a doublet of 52/48 kD. 'Liver-pancreas' antigen was also recognized by 3% PSC and 9% PBC patients but not by normal controls.
1.10 Human Herpesvirus 6 (HHV-6) and Transplantation

1.10.1 Discovery of a New Herpesvirus

Human herpesvirus 6 (HHV-6) was discovered by Salahuddin et al (1986) in a group of patients with lymphoproliferative disorders (323); B cells were readily infected \textit{in vitro} giving rise to cytomegaly and inclusions and the virus was initially called human B-lymphotropic virus (HBLV).

This group showed, in an accompanying paper (324), that HHV-6 has a double-stranded DNA genome larger than 110 Kb in length; this is consistent with herpesvirus classification. HHV-6 has a unique genome and hybridisation work demonstrated a 9 Kb region without homology to the genomes of other herpesviruses (324). Amino acid sequences were derived after DNA sequencing by Lawrence et al 1990 (327); homology with CMV led to β-herpesvirus classification. The ultrastructure of HHV-6 also befits a herpesvirus (325, 326) (see section 1.2.1).

Genomic polymorphisms separate HHV-6 variants A and B (333, 334, 335, 356) which also have different growth patterns, \textit{in vitro} tropism and antigens (immunological distinction) (336).

1.10.2 Biology and Virology of HHV-6

Compared to CMV, relatively little is known about the biology and virology of HHV-6.
The prevalence of HHV-6 varies between 60-95% (328, 329) and infection has been linked with various clinical manifestations. Primary infection with HHV-6 variant B causes exanthem subitum in infants (326, 340, 341, 342). Transmission may occur through saliva; salivary glands harbour virus and this may be a site of latency (337, 338).

HHV-6 may be involved in the progression to acquired immune deficiency syndrome (AIDS) in human immunodeficiency virus (HIV)-positive individuals (342). CD4+ T-lymphocytes are the main carriers of both HHV-6 (339, 340, 334) and HIV and these cells die more rapidly in vitro when co-infected with both viruses (341). In addition, Nicholas et al 1994 (343) demonstrated that several HHV-6 genes encode transactivators and some upregulate the long terminal repeat (LTR) of HIV.

1.10.3 Interaction Between HHV-6 and CMV

Concomitant rises of antibody titres to HHV-6 and CMV have been reported (344, 345, 346, 347, 348). Irving et al 1988 (347) reported simultaneous serological conversion to HHV-6 and CMV in renal and cardiac transplant recipients. This was detected as an appearance of IgM or an eight-fold increase in IgG titre. It was not shown whether CMV and HHV-6 co-infect or whether they generate cross-reactive antibodies.

This group subsequently (344) found that the incidence of seroconversion to HHV-6 among immunocompetent patients was significantly greater in patients with primary CMV (15/25 (60%); p< 0.01) or EBV (17/25 (68%); p<0.01) infections compared to a control group (3/37 (8%)) . Sera was adsorbed with CMV and EBV antigens before testing for HHV-6 specific antibodies; no difference in titre was seen after absorption and it was concluded that antibodies to HHV-6 do not cross-react with CMV or EBV.
However, other reports have shown that antibodies generated against HHV-6 can cross-react with CMV (346, 348, 352). Sutherland et al 1991 (346) reported on 30 liver transplant patients that were HHV-6 antibody negative pre-transplant. Eighteen patients generated antibodies against HHV-6 post-transplant; five such responses were wholly cross-reactive with CMV.

Primary infection with, or vaccination against, CMV in immunocompetent individuals also leads to the generation of antibodies which cross-react with HHV-6 (348); the authors Western blotted CMV proteins and demonstrated that antibodies generated against HHV-6 recognised a 116-kD protein (gene product (gp) 116) and cross-reacted with CMV glycoprotein gB.

1.10.4 HHV-6 Infection After Transplantation
Active HHV-6 infection has been documented after bone marrow (351, 354, 358, 359, 361, 362, 363), renal (350, 355, 357, 364) and liver (349, 346) transplantation.

1.10.4.1 HHV-6 and Liver Transplantation
There have been very few reports of active HHV-6 infection after liver transplantation. The first case was described by Ward et al 1989 (349); diagnosis was made after electron microscopy, DNA hybridisation and immunohistochemistry studies.

The incidence of primary HHV-6 infection in 30 liver transplants was 60% (18/30) in a series documented by Sutherland et al 1991 (346); seroconversion occurred after 4-8 weeks post-transplant. Active HHV-6
infection was monitored by testing for HHV-6 specific antibodies. Clinical observation of patients experiencing active HHV-6, but not CMV, infection showed that HHV-6 may be involved with hepatitis, lung dysfunction, pyrexia and neurological complications.

1.10.4.2 HHV-6 and Bone Marrow Transplantation

A number of reports have documented active HHV-6 infection after bone marrow transplantation; the incidence of which varies between 38-60% (354, 358, 361). In one series (354), all patients with active infection (40% (12/25)) became viraemic between 14-22 days post-transplant.

The largest study to date (361) documents a 60% (36/60) incidence of active infection using PCR surveillance of buffy coat, urine and oral lavage washings. A greater proportion of patients with severe acute graft versus host disease (GVHD) tested PCR positive for HHV-6 (75% (6/8) grade II-IV versus 25% (5/25) grade 0-1; p=0.01); this finding has been supported (359) and parallels the link found between CMV and acute GVHD (see section 1.6.6.3). Restriction fragment length polymorphism (RFLP) analysis of PCR products showed that HHV-6 type B infection occurred in 89% of cases; the preponderance of type B isolates in this setting has been confirmed (358, 363).

Cone et al 1993 (359) propose a link between active HHV-6 infection and the development of acute GVHD (see 361) and pneumonitis. Lung tissue from 15 bone marrow transplant patients (all HHV-6 antibody positive) and 15 controls (14 HHV-6 antibody positive and one negative) was subjected to a single round quantitative PCR. High levels of HHV-6 DNA (>20000 genomes/ 10^6 cells) correlated with a higher incidence of severe GVHD (p=0.023) and were found in significantly more patients with
idiopathic rather than attributable pneumonitis (63% (5/8) versus 14% (1/7); p=0.037); high levels also correlated with a reduced risk of death from pneumonitis (p=0.015) and the authors suggest that HHV-6 may be less pathogenic than CMV in this setting.

The involvement of HHV-6 with pneumonitis was supported by immunohistochemistry of lung tissue (351, 360). Pitalia et al 1993 (360) showed that HHV-6 antigens localize in pneumocytes and infiltrating macrophages. Pneumocyte positivity suggests that HHV-6 may initiate damage rather than reactivate after the infiltration of permissive lymphocytes.

HHV-6 has also been linked with the suppression of bone marrow growth \textit{in vitro} (358, 365) and \textit{in vivo} (362). Carrigan and Knox 1994 (362) studied 15 transplant patients that developed bone marrow suppression; HHV-6 isolation from bone marrow occurred more frequently from patients with idiopathic rather than attributable bone marrow suppression (p<0.01).
1.11 OUTLINE OF THESIS AND HYPOTHESES

1.11.1 OVERALL HYPOTHESIS FOR THESIS

- Herpesviruses, CMV and HHV-6 in particular, may induce or enhance chronic rejection of liver grafts.

1.11.2 HYPOTHESES FOR CHAPTERS 2 AND 3

- Detection of cytomegalovirus (CMV) DNA in serum or urine is more sensitive and specific than conventional tests (Chapter 2).

- Detection of cytomegalovirus (CMV) DNA is associated with development of symptomatic infection (Chapter 3).

The lack of an association of cytomegalovirus infection with chronic rejection, reported by some, may be due to poor test sensitivity. In addition, symptoms associated with episodes of acute rejection and active CMV infection are often similar. Therefore, testing the latter 2 hypotheses was an important prerequisite for testing the overall hypothesis.

1.11.3 HYPOTHESES FOR CHAPTER 4

- CMV infection constitutes a risk for chronic rejection of liver grafts.

Mismatched CMV serology (donor positive/recipient negative) and active CMV infection (tested using the techniques validated in Chapters 2 and 3), persistent active CMV infection and viral load were assessed as risk factors for chronic rejection of liver grafts.

- The tumour necrosis factor-2 (TNF-2) promoter allele is a risk factor for chronic rejection.

- This risk factor synergises with active CMV infection.
1.11.4 HYPOTHESES FOR CHAPTER 5

- Production of antibodies to hepatic artery and bile duct tissue initiates or enhances chronic rejection.

- Active cytomegalovirus (CMV) infection of hepatic artery or bile duct upregulates the expression of target antigens.

- Active CMV infection of liver transplant recipients upregulates the production of antibodies involved in chronic rejection.

1.11.5 HYPOTHESES FOR CHAPTER 6

- Cytomegalovirus (CMV) actively infects vascular endothelial and bile duct epithelial cells and directly initiates chronic rejection at these sites.

- CMV infects hepatocytes and indirectly initiates or enhances chronic rejection.

These 2 hypotheses are not mutually exclusive.

1.11.6 HYPOTHESIS FOR CHAPTER 7

- Human herpes virus 6 (HHV-6) is an important pathogen after liver transplantation and active infection initiates or enhances chronic rejection.
CHAPTER 2

DEVELOPMENT OF A SENSITIVE AND SPECIFIC TEST FOR CYTOMEGALOVIRUS:
DOT-BLOT HYBRIDISATION AND THE POLYMERASE CHAIN REACTION
2.1 Introduction

Cytomegalovirus (CMV) is a major cause of mortality and morbidity in organ transplant recipients (87, 92, 148; see sections 1.5 and 1.6). A number of antiviral drugs are available. Prophylactic administration of the drug ganciclovir (a nucleoside analogue) eliminates CMV disease after transplantation (see sections 1.5.4 and 1.6.5) but this regime has some disadvantages; ganciclovir has a number of adverse side effects including myelotoxicity and treatment is costly. Furthermore, in a placebo controlled trial Boeckh M et al (1996) (437) showed that ganciclovir prophylaxis was a risk factor for late onset CMV disease amongst bone marrow transplant recipients.

A compromise position may be clinically advantageous; early, pre-emptive treatment of patients with active CMV infection with ganciclovir (before the development of clinical symptoms) has been shown to improve patient survival and reduce CMV disease (107, 121). A reliable, specific, sensitive and rapid method for the diagnosis of active CMV infection is needed to allow early, targetted ganciclovir treatment.

This chapter reports development of a dot blot hybridisation test for CMV DNA in urine and polymerase chain reaction (PCR) tests for CMV DNA in urine and serum. Published methods were compared and adapted to give optimal conditions. This chapter also reports the development of quantitative and semi-quantitative PCR tests for CMV DNA in serum and urine. Development of a sensitive and specific test for CMV was a crucial prerequisite for testing the overall hypothesis of this thesis (see section 1.11).

Hypothesis Addressed in Chapter 2

- Detection of cytomegalovirus (CMV) DNA in serum or urine, by hybridisation or PCR, would be more sensitive and specific than conventional tests.
2.2 Materials and Methods

The recipes for some commercially available buffers were not available; such buffers are indicated (TM).

2.2.1 Dot-blot Hybridisation

2.2.1.1 Plasmid Gifts

A number of plasmids containing human CMV DNA were used for dot blot hybridisation. The following were used because they were available from departments within Cambridge; initially, the specificity of these probes was examined to determine their suitability for use in a dot-blot hybridisation test.

pSP64-gB comprised the 3.1 kb Xma III restriction fragment of CMV DNA (AD169 strain), encoding the glycoprotein B gene, cloned into plasmid pSP64 (Boehringer-Mannheim).

pGEM-IE1 comprised the 1.8 kb Hind III- Eco R1 restriction fragment, encoding immediate early genes 1 and 2, cloned into plasmid pGEM (Promega). Both pGEM-IE1 and pSP64-gB were kindly supplied by Professor J G P Sissons (41, 48).

pAT153-HindIII contained the 22.81 kb HindIII restriction fragment of CMV DNA (AD169 strain) cloned into plasmid pAT153.

pRK19-UL75 contained the 2.55 kb Hind III/Sma I restriction fragment of CMV (AD169 strain) DNA, encoding the glycoprotein H gene, cloned into plasmid pRK19.
PING-UL115 contained the 2.1 kb Bam HI restriction fragment of CMV DNA (AD169 strain), encoding the glycoprotein L gene, cloned into plasmid pING14.2. The latter three plasmids were kindly supplied by Professor A Minson (58).

2.2.1.2 Construction of Plasmid pT7/T3-IE.PCR and pT7/T3-IE.PCR.Del

I constructed the plasmids designated as p.IE.PCR and p.IE.PCR.Del from PCR products. p.IE.PCR contained a 293bp fragment of IE gene DNA (AD169 strain). Plasmid pIE.PCR.Del was used to perform quantitative PCR (see section 2.2.2.4) and was identical to the former except for a 50 bp deletion within the IE gene fragment. Recombinant PCR techniques (116) were used to create the 50bp deleted insert; primer sequences remained intact. Both inserts were cloned into plasmid pT7/T3 alpha-18 (Life Technologies).
The Insert Sequence of pT7/T3-IE. PCR

The insert sequence was amplified from the IE1 gene by PCR using 1pg of pGEM IE1 as target DNA. Primer sequences (41) for amplification of a 293 bp region of CMV major immediate early gene 1, exon 4 are shown in figure 2.1.

Figure 2.1: Target DNA, Primers and Oligonucleotide Sequence Used to Amplify a 293 bp Region of the CMV Immediate Early Gene 1

Sequence of CMV IE1 gene, exon 4, target region for PCR (293 bp); primers and oligonucleotide probe, used to confirm specificity, are shown.

```
sense

5'  01  AAGTGAATTG TGTGGGTGTC TGTGCTGCTA TGTCTTAGAG GAGACTAGTG
   51  TGATGCTGGC CAAAGCGCTT CTGATAACCA AGCCTGAGTT TATCAGTGTA
   101 ATGAAGCGCC GCATTGAGGA GATCTGATCG AAGGCTGTG TTG CCCAGTACAT
   151 TCTGGGGGCC GATCCTCTGA GAGTCTGCTG TGCTAGTGTG GATGACCTAC
   201 GGGCCATCGC CGAGGAGGCT GATGAGGGAAG AGGCTATTGT AGGCTAGACT
   251 TTGGCCACCAG CTGGTGTCAG GCCTCTGAT TCTCTGGTGT CAC 3'

antisense
```

Amplification was carried out with reaction buffer™ (Perkin Elmer-Cetus), 2 mM MgCl₂ (Perkin Elmer-Cetus), 200 μM each deoxynucleotide triphosphate (Pharmacia) and 1 μM each primer (Pharmacia). A thermal cycler (Omnigene, Hybaid) was used to incubate this mixture at 94°C for five minutes then 55°C for five minutes. Tubes were then centrifuged briefly, to spin down condensation, before the addition of 2.5 U AmpliTaq (Perkin Elmer-Cetus) and 1 μl of Perfect Match Enhancer (Stratagene) and overlaid with one drop of mineral oil (Sigma). Reaction mixtures were
then subjected to 30 thermal cycles (72°C for 90 seconds, 94°C for 30 seconds, 55°C for 30 seconds) and finally 72°C for 10 minutes.

_The Insert Sequence of pT7/T3-IE. PCR. DEL_

The insert sequence of p.IE. PCR.Del was constructed by a recombinant PCR procedure which is shown over the next five pages in figure 2.2.
FIGURE 2.2
Construction of a 50 bp Deleted Version of the Wild Type Target Sequence for use in Quantitative PCR Experiments.

(a) Primers and Sequence
Sequence of wild type PCR target region (IE1, exon 4 fragment) showing primers for amplification of this sequence (IE.sense and IE.antisense), '3' halves' of recombinant PCR primers and the 50 bp region to be deleted (italics).

```
5' 01 AAGTGAGTTCTGTCGGGTGCTGTGCCTGCTA TGTCTTAGAG GAGACTAGTG
51 TGATGCTGGC CAAGCGGCTCTGATAACCA AGCCTGAGTT TATCAGTGA
101 ATGAAGCCGCC GCAATTGAGGAA GATCTGCGTGAGTGTTTTG CCGATACAT
151 TCTGCCCAGC GATCTCTCTGA GAGCTGTGCTC TCCTAGTG ATGATGACCTAC
201 GGGCCATCCGC CGAGGAGTCA GATGAGAGAA AGGCATTGT AGCCTACACT
251 TTGGCCACCG CTGGTGTCAG TCTCTGAT TCTCTGGTGT CAC
```

Note: The 3' halves of the deletion primers are used to prime PCR; 5' ends were subsequently ligated.

(b) Scheme for Deletion Product Construction
i. Two reactions were set up to amplify the regions to be retained; double stranded PCR products are shown below. (The strands that contain 'linkers' (as shown) were annealed in the next stage of this procedure)

**Reaction 1**: IE.sense/ Del. antisense primers

![Diagram of Reaction 1: IE.sense/ Del. antisense primers]

**Primers**: IE.antisense/ Del. sense

![Diagram of Primers: IE.antisense/ Del. sense]
ii. These two PCR products were run on a 3% agarose gel, eluted and purified using a Qiagen Extraction Kit (Qiagen). Both were the correct size (141 and 104 bp respectively). Products were then denatured (94°C, 5 minutes) and annealed (55°C, 2 minutes) giving the DNA construct shown below:

![DNA construct schematic]

iii. These strands were then 'filled in' using Taq polymerase and dNTP's (same concentration as PCR test; 72°C for 5 minutes). This gives the DNA construct shown below:

![DNA construct schematic]
iv. This DNA was now amplified by PCR using IE gene exon 4 primers which were modified at the 5' ends to carry restriction sites (Eco R1 and Hind III) to enable cloning. The sequences of these primers with restriction site-linkers are shown below;

<table>
<thead>
<tr>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>5' GCGC[<strong>GAATTC</strong>]AAGTGATTCTGTGGGTGCT 3' Eco R1 site</td>
</tr>
<tr>
<td>Antisense</td>
<td>5' GCGC[<strong>AAGCTT</strong>]GTGACACCAGAATCAGAGGA 3' Hind III site</td>
</tr>
</tbody>
</table>

v. Subsequently, deleted PCR products (lanes 2, 3, 4) were electrophoresed, alongside wild type product (lane 6) and 100 bp lambda DNA ladder Gibco; lanes 1, 8), through a 2% agarose gel which is shown below.
Deleted target DNA (143 bp) was produced (lanes 2, 3, 4). However, wild type DNA is also present in these lanes and probably reflects 'carry over' of the wild type DNA used in initial PCR reactions.

vi. The deleted target DNA was eluted, taking care to avoid wild type product, restricted with Hind III and Eco R1 and cloned. This plasmid was designated p.IE.PCR.Del.

vii. After propagation and harvesting, 5µg double stranded DNA was sequenced using a USB Sequenase Version 2.0 DNA Sequencing Kit; the identity of the insert as deleted target DNA was confirmed.
Restriction Digestion of DNA
1µg pT7/T3 DNA or PCR product was combined with 1µl Eco RI, 1µl Hind III (10 units) and restriction buffer B^TM (all reagents Boehringer-Mannheim); reactions were incubated at 37°C for 1 hour.

DNA 'Insert' Fragment Purification/ Agarose Gel Electrophoresis
DNA fragments underwent agarose gel electrophoresis; 8g agarose (Sigma) were added to 400ml TAE buffer (0.04M Tris-acetate, 0.001M EDTA), dissolved after heating by microwave, cooled to 60°C (cool enough to hold; at this point ethidium bromide (Sigma) was added to 0.5µg/ml) and set around a comb to form a 2% gel. The gel was submerged in TAE buffer within an electrophoresis tank and DNA was loaded with gel loading buffer (gel loading buffer (6X stock): 0.25% bromophenol blue (Sigma), 0.25% xylene cyanol FF (Sigma), 30% glycerol (Sigma) in water). DNA was separated by applying a potential difference of 5 V/cm.

DNA fragments were visualised above ultraviolet light; linearised pT7/T3 and PCR product fragments were cut out using a scalpel. DNA was released using a Qiagel Extraction Kit (Qiagen) according to manufacturers instructions.

Ligation
Ligation reactions between vector and 'insert' sequences were set up; 100ng pT7/T3 DNA was added to an equal number of copies of PCR product DNA in 7.5µl water. This solution was warmed to 45°C for 5 minutes (to melt cohesive termini) and cooled on ice before addition of 1µl T4 DNA ligase buffer (10X stock; Boehringer-Mannheim), 1µl 5mM ATP and 0.2µl T4 DNA ligase (Boehringer-Mannheim). This mixture was
overlayed with 1 drop mineral oil and incubated at 16°C overnight before transformation of Escherichia coli (E. coli).

**Transformation of E. coli**

E. coli strain XL1-blue allows selection of recombinant plasmids by complementation of the Lac Z gene. This strain was grown overnight in 2TY medium (1.6 % bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl; pH 7.0) in a shaking incubator. A total of 300μl was used to inoculate 30ml fresh 2TY which was incubated as before for 2 hours (until optical density at 550nm was 0.4-0.5). Cells were centrifuged at 6000g for 10 minutes at 4°C in a pre-cooled rotor and resuspended in 15ml ice-cold 50mM CaCl₂. Cells were now incubated on ice for 15 minutes, centrifuged as before and resuspended in 3ml ice-cold 50mM CaCl₂. The contents of the ligation reaction (10 μl) were now added to 300μl of these 'competent' cells and the mixture incubated on ice for 30 minutes before transfer to a 42°C water bath for 2 minutes (heat shock) and brief incubation on ice. Transformed cells were now incubated in 0.5 ml 2TY at 37°C for 1 hour to allow expression of antibiotic resistance genes. Finally, cells were spread on agar plates and cultured at 37°C.

**Selection of E. coli Transformed with Recombinant Plasmid**

2TY-agar (1.6 % bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl, 1.5% bacto-agar) was made. The mixture was autoclaved and cooled. When cool, 60μg/ml ampicillin was added; plasmid T7/T3 contained an ampicillin resistance gene enabling selection for transformed colonies.

Selection for recombinant plasmids was performed by complementation. pT7/T3 carried the gene Lac Z' (encoding the first 146 amino acids of β-galactosidase) which complemented a fragment produced in E. coli
including strain XL1-blue) to form a functional enzyme upon transformation. Enzyme activity was detected by spreading agar plates with 50μl 2% X-gal (5-bromo-4-chloro-3-indoly-β-D-galactoside) and 10μl 100mM IPTG (isopropyl-b-D-thiogalactopyranoside); blue colonies had enzyme activity. The restriction sites for DNA insertion into pT7/T3 were positioned within Lac Z'; insertion of recombinant DNA prevented production of functional Lac Z'. White colonies (no enzyme activity) indicated successful recombination and were chosen.

2.2.1.3 Plasmid Amplification and Harvesting

Propagation of E.coli and Alkaline Lysis

A total of 500ml of 2TY containing appropriate antibiotic (50μg/ml) was inoculated and incubated at 37°C overnight with shaking. Cells were centrifuged at 6000g for 10 minutes at 4°C and resuspended in 18ml 50mM glucose/ 25mM Tris/ 10mM EDTA (pH 8.0). Next, 40 ml 0.2M NaOH/ 1% SDS was added and the contents mixed gently by inversion and incubated at room temperature for 10 minutes before addition of 20 ml ice-cold 5M potassium acetate/ 11.5% (v/v) glacial acetic acid. The contents were mixed and a white precipitate was seen (this contained E.coli chromosomal DNA) and pelleted by centrifugation (6000g, 15 minutes, 4°C, no brake). The supernatant was carefully removed, added to 0.6 volume isopropanol and incubated at room temperature for 15 minutes. The precipitated plasmid DNA was pelleted (6000g, 15 minutes, room temperature), dried, washed twice with 70% ethanol and dissolved in 3ml water.

Purification of Inserted DNA

Probe DNA was released from plasmid preparations by restriction of flanking sites (described above) by appropriate restriction enzymes (Boehringer Mannheim). Probe and plasmid DNA were separated by
agarose gel electrophoresis and probe DNA was purified from agarose using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturers' instructions.

**Sequencing of Double Stranded DNA**
A total of 5µg plasmid was denatured after addition of 0.1 volume 2M NaOH/ 2mM EDTA and incubation at 37°C for 30 minutes. Next, 0.1 volume 3M sodium acetate (pH 5.0) was added to neutralise before DNA was precipitated by addition of 2.5 volume ethanol and incubation at -70°C for 30 minutes. DNA was then centrifuged (13'000 rpm for 10 minutes), washed twice in 70% ethanol and redissolved in 7µl water. DNA was sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (USB) according to the manufacturers instructions.

**2.2.1.3 A 983 base pair (bp) Probe Derived From PCR Amplification**

**PCR Primers**
Primer sequences for amplification of a 983 bp region of CMV major and immediate early gene 1, exon 4 were 5'-GAGGAGATGAA'GTGTATTGGGC-3' (sense) and 5'-GGCTCAGACTTGACAGACACAG-3' (anti sense).

**Probe Construction**
The probe sequence (denoted IE1 983) was amplified from the IE1 gene by PCR using 1pg of pGEM IE1 as target DNA. Amplification was carried out as described earlier (section 2.2.1.2 ).

The PCR product was 983 (bp) in length and underwent agarose gel electrophoresis before purification using the Wizard PCR Preps DNA Purification System (Promega).
2.2.1.5 Probe Labelling and Purification

Random primed DNA labelling (110) was used to label probes with $\alpha$-$P^{32}$ dCTP (Amersham) or DIG-11-dUTP (Boehringer Mannheim) according to Maniatis et al (1989) (111) and Musiani et al (1992) (112) respectively. $P^{32}$ labelled DNA was purified using a Sephadex G50-column (111) whereas DIG labelled DNA was precipitated with ethanol, centrifuged (13,000 rpm, 10 minutes) and redissolved in 50 $\mu$l of water.

2.2.1.6 Dot blotting

DNA (purified or crude extraction) was blotted on to Zeta-Probe nylon membrane (Bio Rad).

*Dot blotting using the vacuum manifold*

Dot blotting with use of a vacuum manifold was carried out according to manufacturers instructions (Bio Rad); 500 $\mu$l of sample was heat denatured at 100°C for 10 minutes then chilled on ice before being applied to prewetted (distilled water) Zeta-Probe membrane by vacuum pressure. A total of 500 $\mu$l of 0.4 M NaOH was then applied similarly before the membrane was rinsed in 2X SSC (20X SSC stock; 3M NaCl, 0.3M trisodium citrate) and dried at 80°C for two hours (no vacuum).

*Dot blotting by direct application*

Dot blotting directly on to Zeta-Probe was achieved as follows. 500 $\mu$l sample was firstly reduced to 20-50 $\mu$l under vacuum before being heat denatured at 100 °C for 10 minutes then chilled on ice. 20 $\mu$l of sample was applied to the membrane as two 10 $\mu$l aliquots (air drying between applications) before air drying, immersing in 0.4 M NaOH for 10 minutes, rinsing in 2X SSC and then drying at 80°C (no vacuum) for 2 hours.
2.2.1.7 Hybridisation

Pre-hybridisation and hybridisation solutions consisted of 150 μl/cm² 1mM EDTA, 0.25 M Na₂HPO₄ (pH7.2), 7% SDS and 100 μg/ml of sheared, sonicated salmon sperm DNA (Sigma) or 50 μg/ml sheared, sonicated calf thymus DNA (Sigma) plus 50 μg/ml sheared, sonicated E.coli chromosomal DNA. Membranes were pre-hybridised and hybridised at 60°C for one hour and 12 hours respectively. P³² labelled probe DNA was denatured by addition of NaOH to 0.4M for 10 minutes then neutralised with HCl, whereas DIG labelled probe was heat denatured (100°C for 10 minutes) then chilled on ice. Washes with 500 μl of wash buffer/cm² were performed serially to 'low stringency' (2X SSC/0.1% SDS, 10 minutes, 20°C), 'high stringency' (0.1X SSC/0.1% SDS, 10 minutes, 25°C) or 'very high stringency' (0.1X SSC/0.1% SDS, 2 x 10 minutes, 60°C).

2.2.1.8 Visualisation of Hybridisation

**P³² labelled probes**

The extent of hybridisation was visualised by exposure to an automated beta particle counter (Instant imager, Packard) and/or to X-ray film (Kodak).

**DIG labelled probes**

Visualisation was achieved according to manufacturers' instructions with modifications. Membranes were firstly equilibrated in buffer 1 (100 mM) maleic acid, 150 mM NaCl (pH 7.5) twice and then blocked with 1% w/v blocking reagent, 5% marvel in buffer 1 for 12 hours. Anti DIG - alkaline phosphatase was applied at a working concentration of 1:10,000 (75 mU/ml) in blocking solution for 30 minutes before undergoing two 15 minute washes with buffer 1 and equilibration with buffer 3 (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl₂). Luminescent detection
(employing lumigen as substrate) was completed following manufacturers' instructions (Boehringer-Mannheim).

2.2.1.9 Human and Herpesvirus DNA Controls

DNA was extracted from fibroblasts infected with HSV-1, HSV-2, CMV and VZV (all containing $10^6$ pfu) generously supplied by Professor A Minson and from EBV transformed B-cells generously supplied by Dr M Wills.

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 Urine Sample Preparation

Initial crude sample preparation

Urine samples were initially subjected to four rounds of freeze/thaw cycles and cell debris was removed by low speed centrifugation (3000 rpm, 5mins) before 1μl of supernatant, neat or diluted 1/20 with water (161, 113), was subjected to PCR.

Ultrafiltration

Urine was filtered using either Centricon-100 (Amicon) or ultrafree-MC filter units 100k (Millipore) systems following protocols reported by Khan et al 1991 (114) and Miller 1994 (162). Both systems captured protein with molecular weight over 100 kD. A total of 50 μl of redissolved retentate was subjected to PCR without further processing.

PEG precipitation of viral particles

The protocol reported by Yamaguchi et al 1992 (163) was followed with modifications. A total of 1 ml of urine was centrifuged at 6500 rpm for two minutes to remove cellular debris. 750 μl of urine supernatant was mixed
with 750 µl 20% PEG 6000 (Sigma) and 375 µl of 2mM NaCl and incubated on ice at 4°C for 12 to 16 hours, before being centrifuged at 13'000 rpm for 30 minutes. The resulting supernatant was decanted and the tubes recentrifuged (6500 rpm, 2 minutes) to remove supernatant further. The pellet was re-suspended in 200 µl water and 50 µl was subjected to PCR.

2.2.2.2 Serum Sample Preparation

Proteinase K Method

DNA was extracted from serum after Wolf et al (115); 50µl of serum was mixed with 50µl 2X Amplitaq buffer (10X stock; Perkin-Elmer Cetus) before 3 freeze/thaw cycles, and incubation (60°C for 1 hour) with 120 µg/ml proteinase K (Boehringer-Mannheim (20mg/ml stock)). This was followed by heating at 95°C for 10 minutes and centrifugation (12'000 g for 5 minutes) to remove debris. The supernatant was used for PCR amplification.

Qiagen Method

DNA was also extracted from 200µl serum using the Qiamp Blood Kit (Qiagen). A total of 25 µl Qiagen protease (19.23 mg/ml) and 200 µl of buffer AL™ (Qiagen) were mixed with 200µl serum before incubation at 70°C for 10 minutes and addition of 210 µl isopropanol. This mixture was applied to a silicone resin (Qiamp spin column, Qiagen) by centrifugation, washed 3 times with 500µl buffer AW™ (Qiagen). DNA was eluted into 200µl preheated (70°C) water after incubation at 70°C for 10 minutes. This elutant was used for PCR amplification.

2.2.2.3 PCR Amplification of Sample DNA

50µl of prepared sample was added to 46 µl of reaction mixture in a 0.5 ml 'amplitube' (Perkin-Elmer Cetus). PCR of a 293 bp region of CMV
immediate early gene was performed as described earlier (see section 2.2.1.2 and figure 2.1).

Subsequently, 30μl of each amplified product was electrophoresed (60V) in a 2% agarose (Sigma) gel using TAE buffer. Gels were photographed over UV light after staining with ethidium bromide (10 μg/ml).

Measures were taken to prevent contamination; DNA extraction, reaction mix preparation (conducted in a positive pressure laminar flow hood) and product electrophoresis were conducted in separate rooms using separate pipettes, aerosol-resistant tips and reagents. Additionally, negative (water) and positive (plasmid) controls were included in each experiment. The plasmid control (p.IE1.PCR.DEL) contained a deleted version (50 bp) of the product (see figure 2.2) enabling possible tube-to-tube contamination to be identified.

2.2.2.4 Non Competitive Quantitive PCR

Plasmids Containing Wild Type and Deleted PCR Targets
p.IE.PCR (wild type) and p.IE.PCR.Del (deleted) were constructed as described earlier (section 2.2.1.2).

Detection of Wild Type and Deleted Targets
Deleted and wild type products were distinguished by differential mobility upon gel electrophoresis.

An automated fluorescence detection technique was adapted from a recent report (117). PCR was conducted as described above using a fluorescein labelled sense primer. Subsequently, 3μl of each product was loaded onto a 6% non-denaturing polyacrylamide gel and electrophoresed using an ABI
373A automatic DNA sequencer (Genescan 1.1 software). The gel was scanned with a 40 mW argon ion laser at a fixed distance from the wells and subsequent fluorescence was detected by a photomultiplier tube. This data was integrated and the fluorescence intensity was presented graphically for each lane loaded. The amount of product (wild type or deleted) was proportional to the area under the corresponding fluorescence intensity peak.

**Accurate Non-Competitive Quantitation**

For each sample, 1pg of deleted target DNA was added to 50μl sample DNA preparation before 5 doubling dilutions; for each sample 6 wild type: deleted ratios were amplified. After amplification, the quantity of wild type and deleted product in each tube was measured (as described above) and was plotted after regression analysis. Parallel curves indicated that amplification of deleted and wild type products had occurred with equal efficiency and a gradient above zero indicated that the reaction was at the exponential phase at the completion of cycling. The amount of initial wild type DNA was calculated from the ratio wild type: deleted which was derived from the curve.

**Semi-Quantitation**

Semi-quantitative PCR of a single sample was performed in a single tube (without the addition of deleted target molecules); wild type product was measured as described above. Exogenous controls, comprising doubling dilutions of wild type target DNA from 5pg to 0.15pg, were run alongside sample tubes and were used to generate a standard curve from which initial wild type DNA could be calculated.
Comparison of Accurate Quantitative PCR with Semi-Quantitative PCR
Sixteen serum samples taken from 3 bone marrow patients, previously shown to be serum PCR positive, were tested by both accurate quantitative and semi-quantitative PCR.

2.2.3 Detection of Early Antigen Fluorescent Foci (DEAff)
MRC 5 fibroblasts were grown to confluence in shell vials using 500μl minimal essential medium (MEM). Inoculation was performed by replacement of media with 500μl specimen and centrifugation (2500 g for 30 minutes). The specimen was then replaced with 500μl MEM and incubation at 37°C for 24 hours was carried out in 5% CO₂. The monolayer was then washed twice in PBS before being fixed in methanol. Monoclonal antibody to immediate early protein (raised in mouse; Dako) was diluted 1 in 500 with PBS and 50μl applied to the monolayer before incubation at 37°C for 30 minutes. The monolayer was then washed 3 times in PBS before dilution (1 in 50 with PBS) and application of fluorescein isothiocyanate-conjugated anti-mouse antibody (raised in goat; Dako). Incubation was carried out at 37°C for 30 minutes before washing 3 times in PBS, washing once in distilled water, drying and observation of fluorescent foci by microscopy.

2.2.4 Isolation of CMV by Cell Culture
CMV was isolated in MRC 5 fibroblasts which were cultured in minimal essential medium (MEM; Gibco BRL) containing 2% foetal calf serum (FCS; Gibco BRL) in culture-flasks (75 cm² surface area). A total of 100μl specimen was added to confluent fibroblasts in 1ml MEM. Flasks were incubated at 37°C for up to 3 weeks; MEM was replaced every 7 days. Fibroblasts were monitored daily for evidence of CMV-mediated
cytopathic effect; this was observed as focal formation of rounded, giant cells.

2.2.5 Collection and Processing of Clinical Samples

Dot-blot Hybridisation and PCR

Serum and urine samples were collected. Whole blood (5ml) was taken and allowed to clot overnight at 4°C; serum was collected from these blood tubes after centrifugation at 2500 g for 10 minutes and stored at -20°C before use. 10ml urine was collected in a sterile 30 ml tube and stored at -20°C before use.

Isolation Through Cell Culture and DEAFF

Whole blood (10ml) was added to preservative-free heparin (250 units) and stored at 4°C overnight. Buffy coat samples (at the interface between red blood cells and plasma) were then removed using a Pasteur pipette and tested immediately. 10ml urine was collected in a sterile 30 ml tube and tested immediately.

2.2.6 Patients and Testing of Clinical Samples

Clinical samples were collected between January 1993 and January 1995 from liver transplant (n=99) and non-autologous bone marrow transplant recipients (n=35). I endeavored to collect these twice weekly during hospitalisation then at each visit to clinic, until 6 months posttransplant.

Bone marrow transplant patients were then selected from this pool for analysis according to the criteria that samples were successfully collected from them for ≥ 2 months and at a rate of ≥ 1 sample (serum, whole blood
and urine) per week; 17 bone marrow allograft recipients were therefore included in this study.

Liver transplant recipients for in-depth study were selected initially according to the criteria that all provided ≥ 1 sample per week (serum, whole blood and urine) for ≥ 2 months. From this group, all patients that experienced chronic rejection were selected (n=14) and a control group (n=18) followed for a comparable period but comprising patients that did not develop chronic rejection was selected sequentially (according to transplant date).

Samples were tested in parallel by PCR, DEAFF and culture.
2.3 Results

2.3.1 Optimising Dot-blot Hybridisation

2.3.1.1 False Positives Using a Digoxigenin (DIG) Labelled Probe

In the first instance pGEM-IE1 was titrated from 3 ng to 0.7 pg using CMV negative urine and sterile distilled water as carriers. These samples, and negative controls containing no plasmid, were heat denatured and blotted onto Zeta Probe membrane (Biorad) before hybridisation with digoxigenin labelled IE1 probe and colourimetric detection. With water as the carrier pGEM-IE1 could be detected with a sensitivity of 3 pg and water alone appeared negative. However when urine was used as the carrier non-specific reactions were observed (figure 2.3).

FIGURE 2.3 Non-Specific Hybridisation of a Digoxigenin Labelled CMV DNA Probe With Dot-Blotted Urine Using Colourimetric detection

pGEM-IE1 was titrated from 3ng to 0.7pg and applied to the filter using water ('dots' 1-8) or urine ('dots' 9-16) as carriers. Urine was obtained from a CMV antibody negative individual.

'Dots' 9-16 1-8
Experiments were undertaken to identify the factors mediating this non-specific reaction. The experiment described above was repeated whilst firstly omitting the DIG labelled probe (data not shown) and secondly omitting both DIG labelled probe and anti DIG fab fragments (figure 2.4); here blotted urine was being bathed directly with the colourimetric reagents NBT and X-PHOS. In both experiments false positives occurred when urine was used as the carrier; dot-blotted urine interacted directly with the colourimetric substrates NBT and X-PHOS.

**FIGURE 2.4 Dot-Blotted Urine Interacted Directly with the Colourimetric Substrates NBT and X-PHOS but not with Lumigen**

Water ('dot' 9-12) and urine ('dot' 13-16) were dot-blotted and bathed in colourimetric (a) and luminescent (b) detection substrates; a developed filter (a) and autoradiograph (b) are shown. Anti-digoxigenin antibody-alkaline phosphatase conjugate was blotted onto the membrane (omitting the heat denaturation step) as a positive control ('dot' 1-8).

<table>
<thead>
<tr>
<th>(a) Colourimetric</th>
<th>(b) Luminescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Dot'</td>
<td>'Dot'</td>
</tr>
<tr>
<td>1-8</td>
<td>1-8</td>
</tr>
<tr>
<td>9-16</td>
<td>9-16</td>
</tr>
</tbody>
</table>

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The luminescent detection system (LDS) (Boehringer-Mannheim) was investigated. No direct interaction was seen between urine and the LDS substrate lumigen (figure 2.4). However, a small amount of non specific interaction was seen between urine and the anti DIG fab fragments when using the LDS. Therefore, digoxigenin labelling and detection was abandoned in favour of P\(^{32}\).

Summary
- Digoxigen labelling was unsuitable for detection of nucleic acids in dot-blotted urine.

2.3.1.2 Specificity of \(^{32}\)P-Labelled CMV DNA Probes
2.3.1.2.1 False Positives After Testing Clinical Samples
A number of DEAFF positive and DEAFF negative urines (the latter from both healthy CMV antibody negative and positive individuals) were tested using a \(^{32}\)P-labelled IE1 DNA probe. Samples were processed following a method adapted from Kimpton et al 1991 (118). Virus particles were pelleted by ultracentrifugation (40'000 rpm, overnight), digested with proteinase K (0.25 mg/ml, 3 hours, 56°C; Boehringer-Mannheim) and phenol:chloroform extracted twice before subsequent heat denaturation and blotting onto Zeta Probe membrane.

Hybridisation was seen to both DEAFF positive and DEAFF negative urines (see figure 2.5). It was thought that false positives were caused by traces of E.coli chromosomal and plasmid DNA contamination of the probe which may have hybridised to E.coli DNA present in the samples.
FIGURE 2.5 $^32$P-Labelled IE Gene DNA Probe Hybridised with Dot-Blotted DNA from CMV Negative Urine

Doubling dilutions of the following were blotted; 400pg pGEM.IE1 (1-7, dot 8 is water), 400pg linearised lambda DNA (Gibco) (9-15, dot 16 is water), CMV DEAFF negative urine preparations (17-24 and 25-32) and CMV DEAFF positive urine preparations (33-40, 41-48 and 49-56); an autoradiograph is shown. The probe hybridised to blotted pGEM-IE1 (1-7) positive control and to blotted DEAFF positive urine (33-56). However, the probe also hybridised to blotted DEAFF negative urine (dots 17 and 25) i.e. non-specificity was observed.

2.3.1.2.2 Probe Contamination with E. coli Nucleic Acids Led to False-Positive Results

To further test specificity, four conventionally prepared CMV probes (HindIII, gL, gB, gH) were hybridised against CMV, human, E.coli and plasmid DNA. Autoradiography after highly stringent washes showed that all four probes hybridised to E.coli chromosomal DNA and to a lesser extent plasmid DNA; this is shown for the Hind III probe in figure 2.6.
FIGURE 2.6 CMV HIND III D Fragment Hybridised to Human DNA and E. coli DNA Under Conditions of Very High Stringency

Doubling dilutions of linearised E. coli chromosomal DNA (10ng- 80pg; 'dot' 1-8) and human fibroblast DNA (16ng- 500pg; 'dot' 9-14) were applied to membranes; 'dot' 15 comprised 3 x 10^6 plaque forming units (pfu) AD169/ 6ng human DNA and 'dot' 16 comprised 1 x 10^6 plaque forming units (pfu) AD169/ 2ng human DNA. Blots were probed with ^32P labelled CMV HIND III D fragment (a) and human DNA (b), at very high stringency.

<table>
<thead>
<tr>
<th>'Dot'</th>
<th>HIND III D</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>01-08</td>
<td>09-16</td>
</tr>
<tr>
<td></td>
<td>01-08</td>
<td>09-16</td>
</tr>
</tbody>
</table>

CMV HIND III D fragment clearly binds to DNA extracted from CMV infected fibroblasts (dots 15, 16), human DNA (dots 9-14; sensitivity 4ng) and E. coli chromosomal DNA (dots 1-8; sensitivity 300pg). Human DNA binds to DNA extracted from human fibroblasts (dots 9-16; sensitivity 500pg) but does not bind E.coli DNA and, therefore, serves as a good control.
CMV probes (Hind III, gH,gB,gL) were also generated from plasmids using Wizard preparations (Promega). Using this method, contaminating E.coli chromosomal DNA or RNA was not seen upon visualisation after agarose electrophoresis.

Although these probes contained less E. coli DNA there still remained amounts sufficient to produce a signal when hybridised, under stringent conditions, against E.coli chromosomal and plasmid DNA.

Summary
• Probes cloned in E.coli contained contaminating E.coli nucleic acids that hybridised to E.coli DNA present in urine.
• Such probes mediated false positivity and were unsuitable for detection of nucleic acids in dot-blotted urine.

2.3.1.2.3 CMV DNA Hybridised with Human Genomic DNA
In addition, probe HIND III-D hybridised to human DNA (figure 2.6). An attempt was made to further localise the region of human homology present within the Hind D fragment. Restriction using Eco R1 generated four fragments (see figure 2.7) which were purified by gel electrophoresis. These fragments were eluted and labelled ($^{32}$P) before hybridising against CMV and human DNA. Filters were also probed with labelled ($^{32}$P) human DNA as a control. Under high stringency all four Eco R1 fragments of the HIND III D fragment hybridised to human DNA (see figure 2.7). In addition the GL probe was also shown to hybridise to human DNA under stringent conditions.

Summary
• Two CMV probes hybridised to human DNA at high stringency and were unsuitable for screening clinical samples
The HIND III D Fragment of CMV DNA can be Restricted by Eco R1 into 4 Fragments; All 4 Hybridise with Human DNA.

(a)

<table>
<thead>
<tr>
<th>Hind III</th>
<th>3.16 kb</th>
<th>4.186 kb</th>
<th>10.81 kb</th>
<th>4.657 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eco R1</td>
<td></td>
<td></td>
<td></td>
<td>Eco R1</td>
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<tr>
<td>Eco R1</td>
<td></td>
<td></td>
<td></td>
<td>Eco R1</td>
</tr>
</tbody>
</table>

The Hind III D fragment (22.8 kb) can be restricted into 4 fragments by Eco R1. restriction sites and fragment sizes are shown. Fragments were generated by restriction, separated by 3% agarose gel electrophoresis, eluted (Qiagel, Qiagen) and labelled by random priming using α-32P.

(b)

(i) Human  
01-03  04-06  

(ii) (Hind III D)  
01-03  04-06  

(iii) (Eco R1-10.81 kb)  
01-03  04-06  

(iv) (Eco R1-4.66 kb)  
01-03  04-06  

(v) (Eco R1-4.19 kb)  
01-03  04-06  

(vi) (Eco R1-3.6kb)  
01-03  04-06  

Hind III D fragment DNA (16ng, 8ng, 4ng; 'dots' 1-3) and human fibroblast DNA (1ng, 2ng, 4ng; 'dots' 4-6) were dot-blotted. Blots were probed with labelled human, HIND III D fragment and Eco R1 fragment DNA as indicated. Human DNA hybridised with human DNA but not CMV Hind III D fragment and served as a good control. The Hind III D fragment and all associated Eco R1 restriction fragments hybridised with human DNA at high stringency.
2.3.1.2.4 Blocking Experiments

The hybridisation of Wizard preparation (Promega) purified probes against CMV, human and E.coli DNA was repeated but identical filters were prehybridised and hybridised in parallel in the presence of 100 μg/ml sonicated salmon sperm DNA (Sigma) or 50 μg/ml of calf thymus DNA (Sigma) plus 50 μg/ml E.coli chromosomal DNA. The latter regime was used in an attempt to block the hybridisation of traces of E.coli chromosomal DNA, and/or hybridisation to human sequences by probe gL, by competition with excess amounts of unlabelled DNA (figure 2.8). The degree of hybridisation of probes gB, gH and gL to E.coli chromosomal DNA and of gL to human DNA was lessened when the latter regime was used, without altering affinity to CMV DNA, but not eliminated completely. In addition, hybridisation of CMV DNA probes to CMV DNA was reduced when E.coli/calf thymus DNA was used to prehybridise (see figure 2.8).

Summary

• The inclusion of unlabelled E.coli and mammalian DNA during hybridisation did not eliminate false positivity

• Therefore an attempt was made to produce probes without employing plasmid amplification through E.coli.
Halving dilutions of the following were blotted: DNA from CMV infected fibroblasts ($10^6$ pfu AD169/10ng DNA- $1.25 \times 10^5$ pfu AD169/1.25ng DNA; ‘dots’ 1-4), CMV infected fibroblasts ($10^6$ pfu- $10^5$ pfu AD169; ‘dots’ 5-8), E. coli DNA (10-1.25ng; ‘dots’ 9-12), plasmid pSP64 (10-1.25 ng; ‘dots’ 13-16) and human fibroblast DNA (10-1.25 ng; ‘dots’ 17-20). Filters were processed in parallel and prehybridised/hybridised with 100μg/ml salmon sperm DNA (a) or 50μg/ml calf thymus DNA/50μg/ml E.coli DNA (b). CMV probe gL was investigated; E. coli DNA probe was used as a control. Labelled E. coli DNA hybridised specifically with dot-blotted E. coli DNA and served as a good control; less hybridisation was seen when E. coli DNA was included in the prehybridisation/hybridisation solution (b).

Under 'standard' hybridisation conditions (a) CMV DNA probe gL binds to CMV DNA (‘dots’ 1-8), E.coli DNA (‘dots’ 9-12) and human DNA (‘dots’ 17-20). When E. coli/ calf thymus DNA was included in the prehybridisation/hybridisation solution (b) less hybridisation is seen to E. coli chromosomal (‘dots’ 9-12) and human DNA (‘dots’ 17-20). However, hybridisation to CMV DNA (‘dots’ 1-8) is also reduced markedly.
2.3.1.2.5 PCR Probes

A 983bp probe was produced by PCR amplification (IE1 983). In the first instance this probe was investigated for specificity by hybridisation to CMV, human, E.coli chromosomal and plasmid DNA (figure 2.9). Hybridisation was restricted exclusively to CMV DNA. Similar experiments showed that this probe did not hybridise to HSV (types I and II), EBV, VZV or HHV-6 DNA.

**FIGURE 2.9** A CMV DNA Probe (IE1.983) that was Amplified by PCR did Not Hybridise to Human or E. coli DNA

Doubling dilutions of linearised E. coli chromosomal DNA (10ng-80pg; ‘dot’ 1-8) and human fibroblast DNA (16ng-500pg; ‘dot’ 9-14) were applied to membranes; ‘dot’ 15 comprised 3 x 10^6 plaque forming units (pfu) AD169/6ng human DNA and ‘dot’ 16 comprised 1 x 10^6 plaque forming units (pfu) AD169/2ng human DNA. Blots were probed with ^32P labelled CMV IE1 gene PCR product (IE.983), at high stringency.

**Summary**

- A PCR derived probe from the IE gene hybridised specifically with CMV DNA
2.3.1.3 Sensitivity

2.3.1.3.1 Detection of Naked DNA

Initial experiments showed that 10 pg of target DNA (purified 983 bp PCR product) could be detected.

2.3.1.3.2 Liberation of CMV DNA from AD169 infected fibroblasts

A number of sample treatments were compared; each was applied to 5 ml of CMV negative urine (healthy CMV antibody negative donor) spiked with $10^5$ pfu AD169 before blotting a doubling dilution series. Each spiked urine underwent four freeze/thaw cycles then ultra centrifugation (36000 rpm, 2 hours) before treatment. A comparison was also made between direct application of liberated DNA to the membrane and the use of a vacuum manifold. After blotting, each membrane was hybridised with $^{32}$P labelled PCR.1E1.293 and washed stringently before autoradiography (figure 2.10).

Direct DNA application achieved greater sensitivity than manifold application and was most apparent when the phenol:chloroform step was omitted (lanes 1-20; blots a and b). The lower sensitivity achieved by manifold blotting was likely to be due to competition for membrane binding sites between CMV DNA and contaminating proteins. Additionally, for sample treatments involving SDS, without its removal by DNA precipitation (lanes 5-8, 25-28; blots a and b), direct application allowed the spread of this wetting agent therefore enhancing sensitivity. Similarly, separation of DNA from protein contaminants (phenol:chloroform treatment) and SDS (DNA precipitation) did not improve sensitivity when direct application was followed. After ultra-centrifugation, proteinase K treatment was more effective when used in conjunction with SDS (lanes 9-12, 17-28; blots a and b). In situ proteinase

Table 2.1 Combinations of Treatments Compared in Order to Optimise CMV DNA Preparation before Hybridisation (Results Shown in Figure 2.10)

<table>
<thead>
<tr>
<th>'Dots' / Filter</th>
<th>1 hour incubation</th>
<th>Phenol: chloroform</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proteinase k</td>
<td>SDS</td>
<td></td>
</tr>
<tr>
<td>01-04 a</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>05-08 a</td>
<td>NO</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>09-12 a</td>
<td>YES</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>13-16 a</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>17-20 a</td>
<td>YES</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>21-24 a</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>25-28 a</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>01-04 b</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>05-08 b</td>
<td>NO</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>09-12 b</td>
<td>YES</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>13-16 b</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>17-20 b</td>
<td>YES</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>21-24 b</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>25-28 b</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>01-04 c</td>
<td>YES*</td>
<td>YES*</td>
<td>NO</td>
</tr>
<tr>
<td>05-08 c</td>
<td>YES*</td>
<td>YES*</td>
<td>NO</td>
</tr>
</tbody>
</table>

* Performed in situ after blotting

Table 2.1 shows combinations of treatments used to generate Figure 2.10; 'dots' comprise AD169 virions that have been subject (YES) or not (NO) to various treatments and applied to the membrane by 'dotting' with a pipette (D) or by using a vacuum manifold (M).
5 ml CMV negative urine (donated by a healthy sero-negative) spiked with $10^5$ PFU AD169 was ultra centrifuged (36,000 rpm, 2 hours) and the pellet redissolved in 500 μl water. A proportion of samples were now incubated at 37°C for 1 hour with 1 mg/ml proteinase K (Boehringer Mannheim) and 0.1% SDS, phenol:chloroform extracted and ethanol precipitated (-20°C overnight). Other samples received one or more of these treatments or were 'untreated'. Samples were then heat-denatured before direct dot-blotting or use of a vacuum manifold. This scheme is shown in table 2.1 (overleaf). Blots were hybridised with $^32$P-labelled IE.PCR, washed at high stringency and developed using a phosphoimager.
In a further experiment using CMV DEAFF positive urine it was found that ultra centrifugation at 60'000 rpm for two hours or 36'000 rpm for two hours after PEG precipitation overnight was more sensitive than ultra centrifugation (36'000 rpm for two hours with no PEG precipitation) used the preceding experiment. It was also shown that removal of debris by a 3000 rpm centrifugation before ultra centrifugation greatly reduced sensitivity even after the sample had undergone four freeze thaw cycles.

Summary

- The optimal preparation method for urine was shown to be four freeze/thaw cycles and ultra centrifugation (60'000 rpm for two hours) before in vitro proteinase K/SDS treatment, phenol:chloroform extraction, heat denaturation and direct application onto a nylon membrane.

2.3.2 Dot Blot Hybridisation of Clinical Urine Samples

2.3.2.1 Latent Virus Was Not Detected by Dot Blot Hybridisation

A P³² labelled PCR derived probe (IE1 983) was hybridised against clinical samples prepared using the optimised liberation protocol (above). Dot blot hybridisation of two DEAFF positive samples generated positive results but specimens taken from healthy CMV antibody negative or positive individuals were negative; the latter result is important and suggested that latent virus could not be detected with this protocol.

2.3.2.2 Comparison of Dot Blot Hybridisation with Culture and DEAFF Testing

Thirty two urine samples, taken from liver, bone marrow, heart or renal transplant recipients, were screened by dot blot hybridisation. The viral status of these samples was known after testing by the 'gold standard' tests of culture and DEAFF; 20 samples were positive by CMV DEAFF or culture and 12 samples were negative (Table 2.2).
Table 2.2

<table>
<thead>
<tr>
<th>Dot blot +</th>
<th>DEAFF/ Culture +</th>
<th>DEAFF/ Culture -</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>n=32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Samples from 11 patients that were positive by DEAFF or culture were not positive by dot-blot hybridisation. Therefore, the sensitivity of dot blot hybridisation (compared to culture and DEAFF) was only 45%.

However, dot-blot hybridisation may be a useful complementary test when conventional tests fail. This was suggested by the results from five patients who were positive by dot-blot hybridisation but were negative by culture and DEAFF.

Summary
• Hybridisation with a PCR derived probe (IE gene) detected CMV in urine from patients with active CMV infection
• Dot-blot hybridisation has low sensitivity compared to isolation through culture and DEAFF but may serve as a useful complementary test

2.3.3 Optimising Polymerase Chain Reaction (PCR) Amplification of CMV DNA in Serum and Urine

The polymerase chain reaction (PCR) was employed because the sensitivity of dot-blot hybridisation was disappointing. PCR was optimised and examined using a new panel of control samples (the panel used for optimisation of dot-blot hybridisation had been exhausted).
2.3.3.1  Sensitivity
Amplification of doubling dilutions of target DNA (pIEN, see Methods and Materials), followed by gel electrophoresis gave a sensitivity of 0.078 fg which is equivalent to 561 genome copies (figure 2.11).

**FIGURE 2.11**
Single Round PCR of CMV Target DNA Gave a Sensitivity of 561 copies

Lane

Plasmid DNA was titrated giving a target (insert) DNA titration of 10-0.078pg (lanes 1-8); lane 10 is a 100 base pair DNA ladder (Gibco).

2.3.3.2  Specificity
Initially the immediate early (IE gene) PCR reaction was tested for specificity. Amplification of a 293 base pair fragment was seen to be specific for CMV DNA; no amplification of HSV I and II, EBV, VZV, HHV6, human or E.coli chromosomal DNA was seen. CMV specificity was confirmed by southern blotting and hybridisation with an
oligonucleotide probe specific to the region of the IE gene amplified (figure 2.12).

Summary
• Single round PCR of a 293bp region of CMV DNA was sensitive and specific
FIGURE 2.12
Southern Hybridisation Confirms the Identity of a 293 bp PCR Product

PCR products 6 PCR-positive serum samples (lanes 2-7) were electrophoresed alongside a lambda DNA marker (123 bp/Gibco; lane 1) upon a 2% agarose gel and Southern blotted. The blot was probed with an oligonucleotide probe (see figure 2.1) end-labelled with γ-^32^P.

Upon electrophoresis a 'smear' of DNA (length >293bp) was seen in lanes 6 and 7; this DNA hybridised with the oligonucleotide probe (b) but not at very high stringency (c) and is probably a mixture of cellular DNA products, amplified after non-specific priming of the CMV IE gene PCR primers. The 293 bp PCR product was confirmed to be IE1 exon 4 target DNA; lambda DNA did not hybridise.
2.3.3.3 Optimisation of Urine Sample Preparation

Six DEAFF positive urines and four DEAFF negative controls (collected from 2 CMV antibody-negative and two CMV antibody-positive healthy individuals) were collected for investigation. The DEAFF negative urines were PCR negative.

Only one of six DEAFF positive samples was positive after PCR testing of neat, 'freeze-thawed' urine. This lack of sensitivity was shown to be due to the presence of PCR inhibitors in urine. PCR amplification of 0.156 pg p.IEN was inhibited by addition of 2µl 'freeze-thawed' urine from 5/6 urines tested and in contrast, was unaffected by addition of 2µl of the urine sample that was previously amplified successfully (data not shown).

A number of regimes were employed to try to separate CMV particles from PCR inhibitors (see Materials and Methods). After ultra filtration through Amicon or Millipore filters before PCR of the retentate, two out of six samples were PCR positive. A method based on PEG precipitation of viral particles following debris removal by a low speed spin was attempted; four out of six DEAFF positive urines were PCR positive with this extraction technique. CMV specificity was confirmed by southern blotting and hybridisation with an oligonucleotide probe specific to the region of the IE gene amplified.

Summary
- Neat urine contained PCR Inhibitors
- Precipitation with PEG (on ice, overnight), after removal of debris by low speed centrifugation, was the optimal method for urine preparation before PCR

2.3.3.4 Optimisation of Serum Sample Preparation

Serum samples were collected from three liver transplant patients experiencing active CMV infection and from healthy CMV antibody
positive and negative individuals in order to optimise sample preparation.

Samples were subjected to proteinase K based extraction techniques and a silicone capture based kit, Qiamp kit (see Methods and Materials), before PCR. CMV DNA was not amplified from serum samples obtained from healthy individuals (CMV antibody negative and positive) but was amplified from those experiencing acute CMV infection. The Qiamp kit successfully extracted amplifiable DNA from blood and serum (see figure 2.13) whereas the proteinase K based techniques were successful only with whole blood.

**Figure 2.13**
The Qiamp Blood Kit Enabled Extraction of Amplifiable DNA from Serum

Serum from 3 patients with active CMV infection were extracted using a proteinase K based technique (lanes 2-6) or the Qiamp Blood Kit (Qiagen; lanes 7-12). 50µl (lanes 3, 5, 6, 8, 10, 12) or 25µl (lanes 2, 4, 7, 9, 11) of each preparation underwent PCR and products were electrophoresed on a 2% agarose gel alongside a lambda DNA ladder (123bp/ Gibco). Negative (water; lanes 13, 14) and positive (100pg, 50pg pT7/T3-IE.PCR; lanes 15, 16) controls were included and were PCR negative and positive respectively. Quiamp extraction (lanes 07-12) was more efficient than a proteinase K based method and enabled amplification of CMV DNA from all 3 patients.

Furthermore, CMV specificity was confirmed by southern blotting and hybridisation with an oligonucleotide probe specific to the region of the IE gene amplified.
Summary

- The Qiamp Blood Kit efficiently extracted DNA from sera.
- CMV DNA was amplified from serum samples from 3 patients with active CMV infection.

2.3.4 Comparison of PCR with Culture and DEAFF Tests

2.3.4.1 Comparison of Urine PCR with Culture and DEAFF Testing

Eighty nine urine samples collected from a mixed population of liver, bone marrow, heart or renal transplant recipients, were tested for CMV by DEAFF, culture and the optimised protocol for PCR amplification (sample preparation by PEG precipitation); (Table 2.3).

Table 2.3 Urine Testing for CMV by DEAFF or Culture was Compared with PCR

<table>
<thead>
<tr>
<th>URINE</th>
<th>PCR vs DEAFF (n=64)</th>
<th>PCR vs culture (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEAFF</td>
<td>CULTURE</td>
</tr>
</tbody>
</table>
|               | +                   | -                     | +                  | -                   | 'toxic'  | +                  | -                   | 'toxic'
| PCR           | 5                   | 2                     | 0                  | 5                   | 2       | 0                  |
| +             | 5                   | 45                    | 7                  | 1                   | 22      | 15                 |

Compared to DEAFF and culture, urine PCR has sensitivity 50% and 83%, specificity 96% and 92% respectively; 11% and 33% of urine samples 'lost' to DEAFF and culture respectively.
Summary

- PCR of CMV DNA from urine had high specificity but low sensitivity compared to isolation through cell culture or DEAFF

- A significant proportion of urine samples were toxic in conventional culture or DEAFF tests

2.3.3.4 Comparison of PCR of CMV DNA from Serum with Culture and DEAFF Testing of Buffy Coat

89 serum and buffy-coat samples were collected from liver and bone marrow transplant recipients and tested for CMV by PCR and DEAFF; 76 were tested by culture (Table 2.4).

Table 2.4 Buffy Coat Testing for CMV by DEAFF or Culture was Compared with PCR Testing of Serum

<table>
<thead>
<tr>
<th>PCR vs DEAFF</th>
<th>PCR vs culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=89)</td>
<td>(n=76)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>DEAFF</th>
<th>CULTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>65</td>
</tr>
<tr>
<td>PCR</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Compared to DEAFF and culture, serum PCR has sensitivity 100% and 100%, specificity 93% and 84% respectively; 17% and 51% of buffy coat samples 'lost' to DEAFF and culture respectively.
Summary

- PCR of CMV DNA from serum had higher sensitivity than isolation throughuffy
  coat cell culture and DEAFF

- A significant proportion of buffy coat samples were toxic in conventional culture and
  DEAFF tests

2.3.5 Quantitative and Semi-Quantitative PCR

Non-competitive quantitative PCR and semi-quantitative PCR were compared using sequential serum samples from three bone marrow transplant recipients that experienced CMV disease. The samples tested had previously been shown to be positive by qualitative PCR; results are shown in figures 2.14 and 2.15.

![Figure 2.14 Semi-Quantitative PCR for CMV in 3 Bone Marrow Transplant Recipients](image)
Accurate quantitation allowed discrimination over 3.5 logs whereas semi-quantitation discriminated over only 2 logs; the latter test may have underestimated the quantity of CMV present after becoming saturated e.g. case 1/sample 4. In contrast, non-competitive quantitative PCR is not prone to such error because each sample is diluted several times. However, the patterns generated by these two tests were essentially similar. For both tests, the maximum viral load was deemed to be highest in case 1 (CMV pneumonitis), followed by case 2 (CMV retinitis) and lowest in case 3 (pyrexia).

Summary
- Quantitative and semi-quantitative PCR gave similar results for sequential serum samples taken from three patients with active CMV infection.
2.4 DISCUSSION

2.4.1 Conclusions

- The hypothesis that detection of CMV DNA in serum would be superior to conventional detection methods was upheld; serum PCR was more sensitive than culture and DEAFF.

- The hypothesis that detection of CMV DNA in urine would be superior to conventional methods was not upheld.

*Dot-blot Hybridisation*

- The digoxigen labelling system was unsuitable for detection of viral nucleic acids in dot-blotted urine because of the interaction between urine and colourimetric reagents and the anti-digoxigenin antibody.

- Conventional preparation of probes cloned after amplification in E.coli led to contamination with E.coli nucleic acids; these hybridised to E.coli DNA present in urine and mediated false positivity.

- Two CMV probes (Hind III and gL) hybridised to human DNA at high stringency; these probes were unsuitable for detection of viral nucleic acids in clinical samples.

- A PCR derived probe from the immediate early gene of CMV detected dot-blotted CMV DNA specifically.

- Optimal preparation of urine for detection of CMV DNA was as follows; four freeze/thaw cycles and ultra centrifugation (60'000 rpm for two hours) before *in vitro* proteinase K/SDS treatment, phenol:chloroform extraction, heat denaturation and direct application onto a nylon membrane.

- However, dot-blot hybridisation was a poor test for CMV in urine; it had low sensitivity and specificity compared to isolation through culture and DEAFF.
Polymerase Chain Reaction

- Single round PCR of a 293bp region of CMV DNA was sensitive and specific to CMV targets

- Neat urine contained PCR inhibitors that can be partly removed by using a PEG precipitation method.

- The Qiamp Blood Kit efficiently extracted DNA from sera

- PCR of CMV DNA from serum was more sensitive than isolation through cell culture and DEAFF

- PCR of CMV DNA from urine was specific but was insensitive compared to conventional culture and DEAFF

- A significant proportion of urine and buffy coat samples were toxic to conventional culture and DEAFF tests

- Quantitative and semi-quantitative PCR yielded similar results

2.4.2 Digoxigenin Labelling and Dot-blot Hybridisation of Urine
The digoxigenin labelling and detection system may not be suitable for the detection of DNA in crude DNA extractions from clinical material. Dot-blotted urine reacted directly with the colourimetric reagents NBT and X-PHOS. Cells sloughed into urine have endogenous alkaline phosphatase activity; however, enzyme activity should have been destroyed during DNA denaturation (95°C, 5 minutes) and levamisole (inhibitor of human alkaline phosphatase) was not found to be preventative.
False positivity was also seen when lumigen was used as substrate; again, components of the digoxigenin labelling and detection system were involved. It was thought that non-specific interaction between dot-blotted proteins and anti-digoxigenin antibody was responsible and a more robust blocking procedure (5% Marvel, overnight) eliminated this problem.

2.4.3 Contamination of Probes with E.coli Nucleic Acids

It was shown, using $^{32}\text{P}$ labelled probes, that E.coli chromosomal DNA contamination of plasmid derived probes consistently mediated false positive reactions when applied to crude DNA extracted from CMV negative urines. False positivity was reduced but not eliminated using preparations with less chromosomal contamination (Wizard Preparations - Promega) and by including unlabelled E.coli chromosomal DNA in prehybridisation and hybridisation solutions. Separation of the probe (plasmid insert) by electrophoresis eliminates most contamination but will not eliminate E.coli DNA of a similar length.

A number of reports (157, 158, 159) suggest that vector sequence contamination mediates false positive reactions when probing crude DNA extractions from clinical material. Ambinder et al (157) included unlabelled plasmid DNA in prehybridisation and hybridisation solutions and used M13 and lambda bacteriophage vectors. However, this did not eliminate false positive reactions which, in contrast, was shown in this thesis to be mediated by E.coli chromosomal DNA.

2.4.4 Hybridisation of CMV DNA Probes with Human DNA

Further specificity problems were encountered. CMV DNA fragments HIND III D (20 kB) and UL115 (gL gene) were both found to hybridise under stringent conditions to human DNA.
Both HIND III D and E fragments (the latter contains UL115 which is the open reading frame for glycoprotein L (gL)) have been shown to contain regions homologous to human DNA (118, 173; see figure). In both previous papers, the Eco R1 U fragment (4.2 kb portion of Hind III D) did not hybridise with human DNA and I therefore isolated this fragment. In contrast, I found that this fragment did hybridise with human DNA, even under very stringent conditions and therefore sought an alternative.

2.4.5 Dot-blot Hybridisation Using a PCR Derived Probe

A probe derived from PCR of the IE1 gene (exon 4) showed no homology with human, EBV, HSV (I and II) or HHV6 DNA.

The sensitivity of hybridisation to naked target DNA (10 pg) was similar to sensitivities achieved by other workers (174, 175, 176, 177, 158, 159). A method for the liberation of DNA from AD169 particles was optimised; direct application of extracted DNA on to the nylon membrane precluded the need for a DNA precipitation step to separate DNA from SDS and protein contaminants. In my hands, SDS/proteinase K treatment of blotted samples performed \textit{in situ} (following Musiani et al 1992) (112) was less sensitive than methods employing this step \textit{in vitro}. The optimised method was similar to others reported (118, 157, 158).

Dot blot hybridisation was not a good candidate for routine surveillance of urine for CMV; sensitivity was poor and the methods employed were time consuming (each run took four days to perform), tedious and technically demanding.
2.4.6 Optimisation of Polymerase Chain Reaction (PCR) Amplification of CMV DNA from Serum and Urine

Polymerase chain reaction (PCR) amplification of a 293 bp fragment of IE1 gene exon 4 was specific and sensitive; amplification did not occur with E.coli, human or other herpesvirus DNA and 0.078fg (561 genomes) target could be detected after PCR and agarose gel electrophoresis. This sensitivity was comparable with other reports of single round PCR systems (163, 169).

Amplification parameters were optimised by Weideman JTW (41) with modifications for this study; use of Amplitaq (Perkin Elmer) improved sensitivity; this is a recombinant form of DNA polymerase (from *Thermus aquaticus*) with no 3'-5' exonuclease activity and is therefore highly efficient. In addition, sensitivity was increased by the use of Perfect Match Enhancer (Stratagene); this additive destabilizes mismatched primer-template interactions and therefore increased reaction specificity. Therefore, primers and reagents are used almost exclusively for specific amplification of target DNA which leads to enhanced sensitivity.

Serum and samples were efficiently processed using the Qiamp Blood Kit which performed better than a proteinase K based technique. The lack of sensitivity of the proteinase K based technique is due to residual proteinase K activity during PCR according to Tysoe (178); however, I found this an unlikely explanation because proteinase K activity is destroyed at 94°C. Instead, serum proteins may be acting as non-specific, non-enzymatic PCR inhibitors; they are not eliminated by the proteinase K based DNA extraction technique but are eliminated after Qiamp extraction. Amplification was seen only from blood samples collected from individuals experiencing episodes of active CMV infection and this
specificity was further substantiated when testing clinical samples. Other reports have also demonstrated the inability of single round PCR to detect CMV in healthy carriers (167, 183).

2.4.7 Comparison of PCR with DEAFF and Culture Testing of CMV

**Serum**

When serum and buffy coat samples from transplant recipients were tested in parallel by PCR and culture and/or DEAFF respectively, it was shown that PCR of serum was more sensitive than DEAFF or culture. In fact, none of the serum samples that were positive by PCR were also positive by DEAFF or culture. The PCR test was nevertheless shown to be specific after probing with a CMV-specific oligonucleotide and all samples that were PCR positive were taken from patients that were experiencing CMV disease (confirmed by DEAFF and culture testing of urine).

Previous reports have shown PCR testing of whole blood to be more sensitive than conventional cell culture (166, 167, 160, 168, 169, 170) and DEAFF (165, 171, 166, 167, 160, 172, 170). Zipeto 1992 (167) found that a third of blood samples (n=293) taken from a mixed population of transplant recipients, patients with AIDS and newborns tested positive by PCR but negative by DEAFF and culture.

Most of the PCR positive serum samples (13/15) were taken from 2 bone marrow transplant recipients with severe CMV disease (see chapter 3) and it is surprising that samples remained DEAFF and culture negative. It is unlikely that these two patients harboured a strain of CMV with unusual *in vitro* growth characteristics (urine was DEAFF and culture positive) or
one which escaped antibody detection of the DEAFF test (immediate early proteins are highly conserved). It is more likely that obliteration of lymphocytes (cells tropic for CMV in buffy coat) prevented detection. In addition, a high proportion of samples were toxic to the fibroblasts used in the DEAFF test (17% toxic) and culture (51% toxic). A proportion of these 'toxic samples' were taken from patients that were receiving ganciclovir treatment (33% and 31% respectively). Ganciclovir treatment may be partly responsible for the toxicity observed (it is possible that the drugs activity is not wholly confined to viral DNA polymerase and may affect cellular DNA polymerase).

**Urine**

Establishing a reliable PCR test for urine proved difficult because of PCR inhibitors. The presence of PCR inhibitors in urine has been reported (171, 160, 114, 179, 113, 163). Previous workers have attempted to overcome inhibition by ultra filtration (114), dilution (113, 161), nested PCR (113, 114, 164, 184) or PEG precipitation methods (163). Published methods were compared and adapted; PEG precipitation was the most useful. Khan 1991 (114) showed that urea concentrations of 100 mM inhibit PCR but I found that inhibition did not necessarily occur in samples with the highest urea concentrations suggesting that other inhibitors are present (data not shown). This phenomenon has been previously reported (114) but the nature of this inhibition is unclear; ultra filtration and PEG precipitation eliminate aqueous inhibitors but retain protein and lipid which may be responsible.

Urine samples from transplant recipients were tested in parallel by PCR, culture and/or DEAFF. The optimised method only attained sensitivities of 83% and 50% compared to culture and DEAFF respectively. Although
PEG precipitation was the most effective method of separating virus particles from inhibitors, it is likely that this step does not lead to their complete removal which has a deleterious effect upon test sensitivity. However, a high proportion of urine samples were toxic to the fibroblasts used in DEAFF and culture testing. Therefore, despite poor sensitivity, PCR of CMV DNA from urine may be useful when performed in conjunction with DEAFF or culture.

2.4.8 Quantitative and Semi-Quantitative PCR

For accurate quantitative PCR, a control (flanked with identical primer binding sequences) must be included to compensate for variations in PCR efficiency between samples or reaction tubes. To differentiate between sample and control targets previous workers have created restriction sites by in vitro mutagenesis (185), introduced single based permutations and separated products by temperature gradients gel electrophoresis (186), introduced heterologous DNA and differentiated between products by hybridisation (188, 182) or made size differences and separated by gel electrophoresis (117, 187, 182).

In this study a 50 base bed deletion of the wild type sequence was made using a recombinant PCR technique (116) that allows recombination irrespective of restriction site position. Detection and results interpretation was carried out with ease and without use of radioactive isotopes; fluorescein labelled PCR products were separated using an ABI Automated DNA Sequencer.

It is important to amplify a dilution series of each spiked sample; single point analysis is inaccurate because amplification efficiencies may vary
from tube to tube. A dilution series also enables plateauing and or competition between target DNAs to be identified and taken into account. Amplification profiles of wild type (pT7/T3-IE) and recombinant (pT7/T3-IE.Del) targets mirrored each other. Amplification was exponential after 30 cycles and occurred with equal efficiency; these are important pre-requisites for accurate quantitation.

Accurate quantitation was compared with semi-quantitation for serum samples taken from three bone marrow transplant recipients. A number of discrepancies arose because 'saturation' and variations in efficiency between tubes cannot be monitored when performing semi-quantitative PCR. However, both tests generated similar results and ranked these three patients identically (based upon maximum viral load).

It was decided to adopt the semi-quantitative technique for sample screening (see Chapter 4). This assay was less tedious than the accurate quantitative assay which usually had to be repeated several times for each sample tested, using different series of deleted target molecules, to generate parallel curves of positive gradient.
CHAPTER 3

QUALITATIVE AND QUANTITATIVE POLYMERASE CHAIN REACTION (PCR) TESTING FOR CYTOMEGALOVIRUS DNA IN SERUM MAY ALLOW PREDICTION OF SYMPTOMATIC CMV INFECTION
3.1 Introduction

An accurate and sensitive diagnosis of active CMV infection is a crucial prerequisite for testing the overall hypothesis that CMV initiates or enhances chronic rejection of liver grafts.

In addition, to enable targetting of patients for pre-emptive antiviral prophylaxis a accurate prediction of CMV disease must be made before the onset of symptoms.

The results presented in Chapter 2 show clearly that PCR of CMV DNA in serum is more sensitive than buffy coat DEAFF. However, a number of reports show that CMV PCR positivity does not necessarily correlate with symptomatic infection. This has been shown for heart (166), liver (160, 168, 170), kidney (167) and bone marrow (164, 169, 223) transplant recipients. Gerna 1991 (166) reported that, for 14 heart transplant recipients, whole blood PCR positivity alone was never associated with symptoms.

Hypotheses Tested in Chapter 3

- Detection of cytomegalovirus (CMV) DNA by qualitative PCR of serum or urine is associated with development of symptomatic infection

- Detection of CMV DNA by quantitative PCR of serum or urine enables a threshold to be drawn that discriminates between symptomatic and asymptomatic CMV infection
3.2 Patients, Materials and Methods

3.2.1 Liver and Bone Marrow Transplant Recipients and Collection of Clinical Samples

Thirty-two liver and 17 bone marrow transplant recipients were studied. These patients were chosen according to the criteria described in Chapter 2 (section 2.2.6); samples were successfully collected from these patients for ≥ 2 months and at a rate of ≥ 1 sample (serum, whole blood and urine) per week. Sample collection, processing and storage was carried out according to the methods described in Chapter 2 (section 2.2.5).

3.2.2 Laboratory Tests

3.2.2.1 Testing by PCR, DEAFF and Culture

Testing of clinical samples was carried out using optimised PCR tests (see sections 2.2.1.2 and 2.3.3), DEAFF (see section 2.2.3) and isolation through culture (2.2.4) as described in Chapter 2.

3.2.2.2 Serological Testing of Donor and Recipient

CMV specific IgM was detected using a μ-capture ELISA kit (Eurogenetics, Belgium); testing was carried out according to the manufacturers instructions. Briefly, serum samples were applied to microtitre plate wells which had been coated in an anti-μ chain polyclonal antibody. All sample IgM was captured; CMV antigen (whole virus proteins) was then applied before application of CMV specific monoclonal antibody (raised in mouse) which was conjugated to horse radish peroxidase. Colour was developed after addition of substrate (H₂O₂) and chromogen (tetramethyl benzidine (TMB)) and read spectrophotometrically.
CMV specific IgG was detected using an indirect ELISA kit (Diamedix, USA); testing was carried out according to the manufacturers instructions. Briefly, serum samples were applied to microtitre plate wells which had been coated with CMV antigen (whole virus proteins). Anti-human IgG antibody-alkaline phosphatase conjugate was then applied. Colour was developed after addition of substrate (p nitrophenyl phosphate (PNP)) and read spectrophotometrically.

3.2.3 Clinical Data

Clinical was collected by three other workers (Dr Jane Collier, Mr Giles Toogood and Mr Avi Soin); all blind to virological data.

The following symptoms were monitored in liver transplant patients: pyrexia, hepatitis, abnormal liver function test results (LFT's), pneumonia, retinitis. These symptoms were only attributed to CMV after confirmation of CMV infection by laboratory testing. Furthermore, symptoms were not attributed to CMV if another infectious agent (e.g. bacteria) was suspected or, in the case of pyrexia and LFT's, if rejection was concomitant. The posttransplant courses of these patients were thus split into episodes of CMV disease and 'CMV disease-free' episodes; laboratory test results of samples taken during either episode were compared.

The following symptoms were investigated in bone marrow transplant patients: pyrexia, pneumonia, retinitis, leukopenia and thrombocytopenia.

3.2.4 Analysis of Results

Test results were compared with the incidence of disease by calculating the following four statistics. Sensitivity was measured as the proportion of disease episodes (or diseased patients) that tested positive whereas
specificity was measured as the proportion of no-disease episodes/patients that tested negative. Positive predictive value was measured as the proportion of 'test positive' episodes/patients that had disease whereas negative predictive value was measured as the proportion of 'test negative' episodes/patients that were disease-free.

3.3 Results

3.3.1 CMV Disease after Liver Transplantation

3.3.1.1 The Study Group

In this study, 33 patients were investigated. These patients underwent 57 liver transplants, of which 44 were closely monitored for CMV infection and disease for more than 40 days and/or until the graft was lost. This group had a median age of 49.5 years (17-63), received organs from donors with a median age 30.5 (12-56) and had a male: female ratio of 1.5 : 1.

Active CMV infection, detected by PCR, DEAFF, culture and/or seroconversion, occurred in 48% (16/33) patients and 59% (26/44) transplants. The overall incidence of CMV disease was 33% of patients (ie 11/33) and 46% transplants (ie 20/44).

The post-operative courses of 44 liver transplants were split into episodes of CMV disease (n=13) and 'CMV disease-free' episodes (n=36). Five patients experienced more than one episode of CMV disease; three patients experienced two episodes during a single transplant and two patients experienced two episodes of CMV disease over two or more transplants.
3.3.1.2 Prediction of CMV Infection and Disease from CMV Antibody Status

The distribution of donor: recipient CMV antibody status in relation to post transplant active CMV infection and disease are shown in table 3.1 and important points are described below.

Table 3.1 Incidence of active CMV Infection and Disease in Relation to CMV Antibody Status in Liver Transplant Recipients

<table>
<thead>
<tr>
<th>CMV antibody status</th>
<th>Active CMV infection</th>
<th>Proportion symptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+: R+</td>
<td>77% (7/9)</td>
<td>57% (4/7)</td>
</tr>
<tr>
<td>D+: R-</td>
<td>100% (9/9)</td>
<td>78% (7/9)</td>
</tr>
<tr>
<td>D-: R+</td>
<td>50% (3/6)</td>
<td>67% (2/3)</td>
</tr>
<tr>
<td>D-: R-</td>
<td>0% (0/4)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 3.1 shows the proportion of liver transplants (n=28) that involved active CMV infection in relation to CMV antibody status of donor (D) and recipient (R) and the proportion of active CMV infections that were symptomatic (proportion symptomatic). NA= not applicable.

Mismatching for CMV status (i.e. donor CMV antibody positive and recipient CMV antibody negative) was associated with a higher incidence of active infection compared to that observed for other combinations (100% (9/9) versus 53% (10/19); p=0.01).

However, the proportion of active CMV infections that were symptomatic was similar between 'mismatch positive' and 'control (other combinations)' groups (78% (7/9) versus 60% (6/10)).

Summary

- Identification of 'mismatched' donor/recipient combinations allowed retrospective prediction of active CMV infection but not of the proportion of active infections that were symptomatic.
3.3.1.3 Is Qualitative Polymerase Chain Reaction (PCR) Testing of CMV DNA a Clinically Useful Test?

Single Round PCR and Prediction of CMV Disease

Four patients were CMV antibody negative and received grafts from CMV antibody negative donors and were therefore not at risk of CMV infection since they also received CMV negative blood products. All samples taken from these patients, ie 39 serum and 26 urine taken over a median of 97 days (range 69 to 129 days), were CMV negative by PCR.

The post-operative courses of 44 liver transplants were split into episodes of CMV disease (n=13) and 'CMV disease-free' episodes (n=36) as described earlier; the former were defined as episodes of pyrexia, hepatitis, abnormal liver function test results (LFT's), pneumonia or retinitis which correlated with active CMV infection by laboratory testing. Correlation was made between positive PCR and/or DEAFF results and CMV disease at each assessment; results are given in tables 3.2 and 3.3 and important points are described below.
Table 3.2

<table>
<thead>
<tr>
<th></th>
<th>SERUM PCR</th>
<th>URINE PCR</th>
<th>URINE DEAFF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
<td>NEGATIVE</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>CMV DISEASE</td>
<td>11</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>NO CMV DISEASE</td>
<td>2</td>
<td>30</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3.2 shows the distribution of qualitative serum and urine PCR and urine DEAFF results with episodes of CMV disease and 'disease-free' episodes. The sensitivity, specificity, positive predictive value and negative predictive value were calculated for each test from these figures and are given in table 3.3.

Table 3.3

<table>
<thead>
<tr>
<th></th>
<th>SERUM PCR</th>
<th>URINE PCR</th>
<th>URINE DEAFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SENSITIVITY</td>
<td>85%</td>
<td>56%</td>
<td>75%</td>
</tr>
<tr>
<td>SPECIFICITY</td>
<td>94%</td>
<td>81%</td>
<td>83%</td>
</tr>
<tr>
<td>POSITIVE PREDICTIVE VALUE</td>
<td>85%</td>
<td>45%</td>
<td>55%</td>
</tr>
<tr>
<td>NEGATIVE PREDICTIVE VALUE</td>
<td>94%</td>
<td>87%</td>
<td>92%</td>
</tr>
</tbody>
</table>

statistical parameters for qualitative PCR of serum and urine and for urine DEAFF in relation to CMV disease episodes (data from table 3.2)
Note that the total number of 'CMV disease' (n=13) and 'CMV disease-free' (n=36) episodes are not shown in table 3.2 because serum and urine samples were not always available.

Qualitative PCR for the detection of CMV DNA from serum attained higher values of sensitivity, specificity, positive predictive value and negative predictive value when compared to urine PCR and urine DEAFF respectively (see table 3.3). All episodes of CMV disease were associated with serum and/or urine PCR positivity.

**Timing of Qualitative PCR Positivity**

Serum PCR was a specific and sensitive indicator of CMV disease but unless CMV is detected before the onset of symptoms then pre-emptive antiviral prophylaxis is impossible.

For the 44 liver transplants studied, initial serum PCR positivity occurred at a median of 38 days posttransplant (range 0-90 days) and initial urine PCR positivity also occurred at a median of 38 days posttransplant (range 3-114 days).

For each episode of CMV disease, the time of initial PCR positivity in days posttransplant was subtracted from the time of onset of symptoms in days posttransplant and expressed as days before or after the onset of symptoms.

Detection of CMV by qualitative PCR of serum occurred at a median of 3 days before the onset of symptoms (range 12 days before to 14 days after) and before the onset of symptoms in 70% (7/10) patients with CMV disease. This suggests that qualitative PCR of serum may be clinically useful.
In contrast, urine PCR positivity occurred at a median time of 12.5 days after the onset of symptoms (range 11 days before to 20 days after) and preceded symptoms in 60% (3/5) patients.

**Duration of PCR Positivity**

The duration of serum and urine PCR positivity during episodes of CMV infection was correlated with the occurrence of disease. There was no difference between the duration of PCR positivity in symptomatic or asymptomatic episodes.

The median duration of serum PCR positivity was 15 days during symptomatic infection (range 9-100) and 12 days (range 3-53) during asymptomatic CMV infection; the patient with serum PCR positivity of 100 days is presented as a case study later (see case study 1).

Urine PCR positivity occurred for similar lengths of time during symptomatic (median 11 days (range 7-37)) and asymptomatic (median 25 days (range 14-49)) CMV infection.

**Summary**

- Qualitative PCR of CMV DNA from serum had high sensitivity and specificity in relation to CMV disease and was more sensitive and specific than qualitative PCR or DEAFF testing of urine

- Serum and urine PCR positivity occurred before the onset of symptoms in 70% and 60% of CMV diseased patients respectively

- Therefore, serum PCR is a sensitive and specific test for CMV disease that may predict the onset of CMV disease

- The duration of serum or urine PCR positivity did not discriminate between asymptomatic and symptomatic CMV infection
3.3.1.4 Is Semi-Quantitative Polymerase Chain Reaction (PCR) Testing of CMV DNA a Clinically Useful Test?

Only samples that were positive by qualitative PCR were subject to semi-quantitative PCR; this was performed alongside exogenous plasmid controls (see section 2.2.2.4) which were used to plot a standard curve to enable copy number to be inferred for each sample tested.

Peak Viral Load and Prediction of Disease in Patients with active CMV Infection

The results generated were plotted for each patient alongside clinical details. After plotting semi-quantitative serum PCR results, I was able to retrospectively, draw an arbitrary threshold of $2.55 \times 10^5$ genomes which divided perfectly the asymptomatic episodes of active CMV infection from the symptomatic. Such a threshold could not be drawn for semi-quantitative urine PCR.

The distribution of episodes of asymptomatic and symptomatic CMV infection using the arbitrary threshold is given in table 3.4.
TABLE 3.4

CORRELATION OF VIRAL LOAD WITH THE PRESENCE OR ABSENCE OF CMV DISEASE DURING EPISODES OF ACTIVE CMV INFECTION

<table>
<thead>
<tr>
<th></th>
<th>SERUM</th>
<th>URINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;2.55 x 10⁵*</td>
<td>&gt;2.55 x 10⁵*</td>
</tr>
<tr>
<td>EPISODES WITH DISEASE</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>EPISODES WITHOUT DISEASE</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>(p&lt;0.000006)</td>
<td>(N.S.)</td>
<td></td>
</tr>
</tbody>
</table>

The number of episodes with peak viral loads above or below the arbitrarily drawn threshold value are shown; episodes of active CMV infection are grouped according to presence or absence of symptoms. The sensitivity, specificity, positive predictive value and negative predictive value for quantitative PCR of CMV DNA in serum, in relation to the occurrence of CMV disease during episodes of active CMV infection, were uniformly 100%; quantitative PCR of CMV DNA in urine gave statistic values of 43%, 14%, 33% and 20% respectively. The number of peaks plotted and analysed does not equal the number of episodes of active CMV infection; analysis was performed on each peak to allow vigorous examination of specificity.

Viral Load in Serum Reaches the Threshold Value Before or Simultaneously with the Onset of CMV Disease

Eleven episodes of symptomatic CMV infection were associated with a CMV load in serum that exceeded an arbitrary threshold; the time taken to exceed this threshold and the time of CMV disease was compared for seven of these episodes (clinical data regarding the timing of CMV disease was insufficient for four episodes).
Semi-quantitative PCR may enable preemptive antiviral prophylaxis; viral load in serum reached the arbitrary threshold of $2.55 \times 10^5$ genomes/ml before or simultaneously with the onset of disease in 4/7 and 3/7 episodes respectively. The threshold was exceeded before disease onset by a median of 4 days (range 0-10 days).

**CMV Disease and "Cumulative Viral Load"**

Measuring the area under plots of viral load with time enabled calculation of the "cumulative viral load" for each episode of active CMV infection; this parameter takes into account viral load and duration of infection.

After plotting "cumulative viral load" for serum PCR results, I was able to draw an arbitrary threshold of $3.8 \times 10^6$ genomes which divided perfectly the asymptomatic episodes of active CMV infection from the symptomatic. Such a threshold could not be drawn for "cumulative viral load" for urine PCR.

The distribution of episodes of asymptomatic and symptomatic CMV infection with this arbitrary threshold is given in table 3.5.
Table 3.5

Correlation of 'cumulative' viral load with the presence or absence of CMV disease during episodes of active CMV infection in liver transplant recipients

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;3.8 x 10⁶⁺</td>
<td>&gt;3.8 x 10⁶⁺</td>
</tr>
<tr>
<td>Episodes with disease</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Episodes without disease</td>
<td>(p=0.00003)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(N.S.)</td>
<td></td>
</tr>
</tbody>
</table>

The 'cumulative' viral load of each episode of active CMV infection was obtained after calculating the area under the plot of viral load against time. Table 3.5 shows the 'cumulative' viral load of each episode, in relation to the threshold value; episodes of active CMV infection are grouped according to presence or absence of CMV-related symptoms. The sensitivity, specificity, positive predictive value and negative predictive value of cumulative viral load of CMV DNA in serum, in relation to the occurrence of CMV disease during episodes of active CMV infection, are uniformly 100%; cumulative viral load of CMV DNA in urine is not clinically useful (statistic values are 50%, 71%, 50% and 71% respectively).
An arbitrary threshold amount of "cumulative viral load" in serum (3.8 x 10^6 genomes) was able to discriminate between symptomatic and asymptomatic episodes of active CMV infection. This parameter improved the specificity of PCR of CMV DNA in serum; specificity and positive predictive value were both 100%. In contrast, this was not shown for "cumulative viral load" for urine PCR.

By definition, 'cumulative viral load' cannot be calculated before the onset of symptoms and is therefore not useful for diagnosis. However, this parameter is a measure of the total amount of CMV replication and here confirmed that CMV replication caused the observed disease episodes.

Summary

- Semiquantitative PCR enabled an arbitrary threshold to be drawn for peak viral load values and "cumulative viral load" in serum; all symptomatic episodes had peak values above this threshold and all asymptomatic episodes had peak values below it

- Such thresholds could not be drawn for urine PCR

- Furthermore, this threshold was exceeded before or simultaneously with the appearance of symptoms for all symptomatic episodes analysed

3.3.2 CMV Disease After Allogeneic Bone Marrow Transplantation

3.3.2.1 The Study Group

In this study, 17 patients were investigated; 13 patients received marrow from HLA-identical siblings and four from HLA-identical unrelated donors. This group had a median age of 38 years (22-52) and had a male: female ratio of 1.5: 1.
3.3.2.2 CMV Infection and Disease

Three recipients were CMV antibody negative and received marrow from CMV antibody negative donors; these patients were not at risk of CMV infection and all samples (40 serum, 22 urine) tested negative by PCR.

CMV infection, positive by serum PCR and/or urine PCR or DEAFF, occurred in 24% (4/17) patients, of whom three developed CMV disease.

The relationship between donor and recipient CMV antibody status, active CMV infection, PCR positivity is shown in table 3.6. Two transplants were performed between donor and recipient with unknown CMV antibody status; neither hosted active CMV infection.

Table 3.6

Incidence of CMV Infection and Disease in Relation to CMV Serology in Bone Marrow Transplant Recipients

<table>
<thead>
<tr>
<th>CMV SEROLOGY</th>
<th>n</th>
<th>ACTIVE CMV INFECTION</th>
<th>CMV DISEASE</th>
<th>URINE POSITIVE</th>
<th>SERUM POSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+: R+</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D+:R-</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D-:R+</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>D-:R-</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 8 shows the number of transplants (n=17) involving active CMV infection (serum and/or urine positivity by PCR) and CMV disease. Two patients of unknown CMV antibody status did not host active CMV infection.

R=recipient, D=donor, n=number.

All three patients that developed CMV disease were CMV antibody positive pretransplant and received marrow from CMV antibody negative
All three patients that developed CMV disease were CMV antibody positive pretransplant and received marrow from CMV antibody negative donors; all three tested positive by serum and urine PCR. All three patients received low dose ganciclovir prophylaxis. A fourth patient (recipient negative and donor positive CMV serology, no ganciclovir prophylaxis) developed active CMV infection (urine positive only) but not CMV disease.

Timing of PCR positivity and disease and viral load in relation to disease is presented in the form of case studies (see cases 4 to 6; figures 3.4 to 3.6). All of the patients with symptomatic infection (n=3) had serum viral loads above the arbitrary threshold of $2 \times 10^5$ genomes/ml that was established using semi-quantitative data from liver transplant recipients. This is further evidence that this threshold may be clinically useful.

### 3.3.2.3 Active CMV Infection and Acute Graft Versus Host Disease

Thirteen patients developed acute graft versus host disease (GVHD); 4/13 also developed active CMV infection. In contrast, none of the four patients that did not develop acute GVHD developed active CMV infection. The link between acute GVHD and CMV remains controversial and is discussed later.

**Summary**

- 24% (4/17) bone marrow transplant recipients developed active CMV infection
- Three of whom were recipient CMV antibody positive: donor CMV antibody negative; all three developed CMV disease. One patient (recipient CMV antibody negative: donor CMV antibody positive) developed asymptomatic CMV infection.
3.3.3 Case studies

Case studies are shown and described in figures 3.1 to 3.6. Serum and urine samples were tested throughout the post-transplant course; qualitative and semiquantitative PCR results are shown. Note that semiquantitation of CMV is illustrated using either 1000 or 10000x genomes/ml as the y-axis scale, according to the peak load of the case.
This case is a liver transplant mismatched for CMV status (ie donor +, recipient -); serum was initially positive on day 36 and remained positive for 108 days until day 144. Initial serum positivity occurred 6 days before symptoms (day 42; pyrexia, hepatitis) which corresponded with a large rise in serum viral load. The threshold of $2 \times 10^5$ viral genomes/ml was reached simultaneously with the onset of symptoms (d42); serum viral load rose to a peak value on day 46. This case did not clear CMV after ganciclovir treatment; however, treatment did reduce viral load which reached levels undetectable by semiquantitative PCR on day 54 (11 days after treatment). Serum viral load also exceeded the threshold value between 70 and 96; symptoms of acute CMV infection were not observed but the patient suffered hepatic artery thrombosis. Urine positivity and load did not correlate with disease.

See also Appendix 1; Patient 29
This case is also a liver transplant mismatched for CMV status (i.e. donor +, recipient -); serum was initially positive on day 26 and therefore occurred 12 days before symptoms (day 38; pyrexia, deteriorating liver function tests) which corresponded with a large rise in serum viral load. The threshold of $2 \times 10^5$ viral genomes/ml was reached on day 29; this was 9 days before the onset of symptoms and rose to a peak value on day 36. This case cleared CMV after ganciclovir treatment; the apparent fall in viral load before ganciclovir is probably a misnomer; serum samples were not available during this period. Urine positivity and load did not correlate with disease.

See also Appendix 1; Patient 2
In this case, liver transplant recipient and donor were both CMV seropositive; serum viral load exceeded the threshold of $2 \times 10^5$ viral genomes/ml on day 23; this was 15 days before the onset of symptoms (d38) which coincided with the peak in serum viral load. Urine positivity occurred after serum positivity and 9 days after the onset of symptoms (d47).

See also Appendix 1; Patient 21.
This case is a bone marrow transplant (donor -, recipient + CMV serology); serum was initially positive on day 67 and therefore occurred concomitantly with the onset of symptoms (pyrexia) but 25 days before diagnosis of CMV pneumonitis which corresponded with a large rise in serum viral load. The arbitrary threshold of $2 \times 10^5$ viral genomes/ml was also exceeded on day 67. Urine load did not correlate with disease. Buffy coat DEAFF was performed regularly after transplantation; of 13 samples tested 12 were negative and 1 was toxic to the test. Ganciclovir therapy was initiated on day 67 but this patient did not respond initially and died 126 days posttransplant; interestingly this patient cleared CMV 19 days before death.

See Appendix 2: Patient 1
CASE 5
BONE MARROW TRANSPLANT
RECIPIENT

This case is also a bone marrow transplant from a CMV serology negative donor to a CMV negative recipient; serum was initially positive on day 45 and therefore positivity occurred 85 days before symptoms (day 130; CMV retinitis). The threshold of 2 x 10^5 viral genomes/ml was also reached on day 45. Buffy coat DEAFF was performed regularly after transplantation; of 29 samples tested all were negative. This patient appeared to clear CMV but urine PCR and DEAFF positivity was detected on day 100 until day 118 and CMV retinitis was diagnosed on day 130; CMV was subclinical until the diagnosis of CMV retinitis. This patient responded clinically to ganciclovir treatment (initiated on day 130) but died on day 309 from multi focal leucoencephalopothy.

See Appendix 2; Patient 2.
CASE 6
BONE MARROW TRANSPLANT RECIPIENT

This case is also a bone marrow transplant from a CMV serology negative donor to a CMV negative recipient; serum was initially positive on day 65. The threshold of $2 \times 10^5$ viral genomes/ml was also reached on day 65; this was 6 days after the onset of symptoms (pyrexia). However, samples were not available immediately after the onset of symptoms. This case cleared CMV after ganciclovir treatment from day 59 to day 66. Urine was not positive by PCR. Buffy coat DEAFF was performed regularly after transplantation; of 19 samples tested 15 were negative and 4 were toxic to the test.

See Appendix 2; patient 3.
3.4 DISCUSSION

3.4.1 Conclusions

- The hypothesis that CMV detection by PCR was associated with the development of symptomatic infection was upheld for serum PCR.

- The hypothesis that semi-quantitative PCR allowed an arbitrary threshold to be drawn that discriminated between asymptomatic and symptomatic episodes of CMV was also upheld for serum PCR.

- Neither hypothesis was upheld for urine PCR.

3.4.1.1 Liver Transplant Recipients

Serology
- Pre-transplant serology for CMV identified patients at risk of CMV disease and may therefore be clinically useful.

Serum PCR of CMV DNA
- Qualitative PCR of CMV DNA from serum may be clinically useful; it had high sensitivity and specificity in relation to CMV disease and positivity occurred before the onset of symptoms in 70% cases.

- The duration of serum PCR positivity did not discriminate between asymptomatic and symptomatic CMV infection.

- Semiquantitative PCR of serum may be clinically useful; arbitrarily drawn thresholds for peak viral load values and "cumulative viral load" discriminated between symptomatic and asymptomatic infections.

Urine PCR of CMV DNA
- Qualitative and semiquantitative urine PCR and urine DEAFF were less clinically useful than serum PCR.

3.4.1.2 Bone Marrow Transplant Recipients
- CMV is an important pathogen after bone marrow transplantation.
3.4.2 Incidence of Active CMV Infection and Disease

The incidence of active CMV infection and disease after liver transplantation (48% and 33%) is consistent with that reported in other centres (93, 94, 43, 96, 97, 99). The incidence after bone marrow transplantation (24% and 18%) is lower than most reports (109, 110, 111). This may be due to the policy at Addenbrooke's NHS Trust to administer low-dose ganciclovir prophylaxis to all bone marrow transplant patients at risk of CMV disease (ie donor or recipient CMV antibody positive).

3.4.3 CMV Serology Identified Patients at Risk of Active CMV Infection

Mismatching for CMV serology (donor positive/recipient negative) is an important risk factor for active CMV infection after liver transplantation. Here, mismatched transplant recipients experienced significantly higher incidences of active CMV infection (100% versus 53%) compared to non-mismatched controls. Similar results have been reported by other groups (43, 93, 94, 96, 98, 99, 227). The findings of Schmidt et al (1993) (227) mirror the results presented here; mismatching was associated with significantly increased active CMV infection (83% versus 48%).

In contrast, all 3/17 bone marrow transplant recipients that developed CMV disease were CMV antibody positive and received marrow from a CMV antibody negative donor. In this scenario, recipient immune responses to CMV are ablated before recipients acquire the donor immune system which is naive to CMV. Similar results have been found by other workers (141, 230, 241, 247). Transplantation of marrow from CMV antibody positive donors into CMV antibody negative recipients results in abortive CMV infection. De Gast (1992) (259) described four such patients; whole blood PCR positivity and granulocyte pp65 antigen expression...
occurred transiently but buffy coat cultures were consistently negative and seroconversion did not occur. It is likely that case 6 of this study is an example of such an abortive CMV infection.

After either liver (donor CMV antibody positive/recipient negative) or bone marrow transplantation (donor CMV antibody negative/recipient positive), recipients that harbour CMV but are immunologically naive are at high risk of active infection and disease. In addition, primary cellular immune responses to CMV are delayed due to immunosuppression. In contrast, CMV antibody positive recipients are primed to mount specific cellular and humoral immune responses to CMV and are able to do so despite immunosuppression.

The importance of such responses in combatting active CMV infection has been demonstrated (65, 260). Reusser et al (1991) (260) studied 20 recipients of marrow from CMV antibody positive donors; 10 recipients were able to mount cytotoxic lymphocyte (CD8 +) responses when challenged in vitro with CMV infected autologous fibroblasts. None of these 10 patients developed CMV pneumonitis whereas 6/10 patients that did not mount an in vitro response died from CMV pneumonitis. Indirect evidence was provided by Fox et al (1988) (98); recipient CMV antibody positivity was not protective when liver transplant recipients were subjected to heavy immunosuppression (cyclosporin-A and anti-thymocyte globulin).

However, if mismatching was avoided then a large number of transplants would be excluded.
3.4.4 Qualitative PCR and Prediction of CMV Disease

The finding that all serum and urine samples taken from CMV antibody negative recipient and donor were PCR negative was an important indicator of specificity. This result is typical of reports made since the implementation of CMV-negative blood product use after transplantation (261).

Some reports have suggested that PCR of whole blood correlates relatively poorly with disease (164, 166, 168, 169, 171, 180, 227); the work presented here shows that detection of CMV in serum and quantitation led to high specificity.

This study shows that PCR of CMV DNA from serum is a sensitive (88% (14/16)) and specific (96% (44/46)) indicator of CMV disease episodes in liver and bone marrow recipients. Two out of 16 episodes of CMV disease in liver transplant recipients did not test positive by serum PCR; it is possible that CMV infection was localised in the kidney (both episodes were associated with urine CMV positivity). It is also possible that serum positivity was transient and occurred when samples were not taken; CMV positivity of serum and urine often differs temporally (see below). Conversely, on two occasions serum positivity was not associated with disease. In both cases, CMV positivity occurred very early (within 1 week) after re-transplantation for chronic rejection (see chapter 4).
transplant recipients by Patel et al (1994) (170) showed that serum PCR could detect active CMV infections with 92% sensitivity and 100% specificity; CMV disease was detected with 100% sensitivity but specificity was only 57%. Patel et al (1994) (170) do not quote sensitivity with respect to naked target DNA but their test is probably more sensitive than the test used in this study because they perform Southern hybridisation after amplification. In contrast, I report 88% sensitivity and 94% specificity; it is possible that there is a trade-off between sensitivity and specificity.

CMV is a cell-associated virus (see Introduction) and only enters serum after lytic infection; abortively infected cells are not detected. In addition, phagocytosed material is not detected by serum PCR.

3.4.5 Semi-Quantitative PCR and Prediction of CMV Disease

Semi-quantitation also improved the specificity and positive predictive value of the test used in this study; all serum samples with viral loads greater than an arbitrarily drawn threshold were associated with symptomatic CMV infection.

This threshold was drawn after plotting of semi-quantitative results; the ability to draw such a threshold suggests that semi-quantitative PCR may be useful but this needs to be confirmed in a larger prospective study.

Quantitative PCR of CMV DNA has been attempted previously (181, 182, 183, 271, 273) and successfully discriminated between asymptomatic and symptomatic CMV infection in renal (271) and liver (273) transplant recipients.
Mutimer et al (1995) (273) have recently reported monitoring 32 liver transplant recipients by quantitative PCR of buffy coat. A 21bp-deleted standard was included in each tube and the initial ratio wildtype:standard was calculated after measurement of PCR products using a rapid hybridisation technique. The results were presented as CMV genomes/1.5 x 10^5 cells; peak load varied between 8.4 x10^4 and 1.2 x10^6 for three patients with symptomatic infection and between 950 and 2.8 x10^5 for asymptomatic patients. In contrast, the results presented in this thesis do not demonstrate overlap of viral loads between symptomatic and asymptomatic; this is possibly due to the specificity afforded through testing serum rather than buffy coat.

Schafer et al (271) used competitive nested PCR's to quantify the number of copies of CMV gB gene DNA per 10^6 B-globin genes present in PBL; internal controls, each differing 3bp from wild type (created by recombinant PCR), were generated for CMV and B-globin targets. Samples were spiked, in parallel, with 50 and 10^4 copies respectively before amplification and products were quantified by temperature gradient gel electrophoresis (TGGE). For serial PBL samples, taken from 17 renal transplant recipients, quantitation discriminated between symptomatic and asymptomatic infection.

Rawal et al 1994 (183) compared two semi-quantitative nested-PCR tests; endpoint titration, visualised after agarose gel electrophoresis, and a method employing measurement of product by scintillation proximity assay (SPA) with comparison to exogenous positive controls. These two methods had comparable sensitivity and specificity. CMV DNA quantities in whole blood samples taken from 6 bone marrow transplant recipients experiencing active infection accurately mirrored infection and the effect
of subsequent treatment but could not discriminate between asymptomatic and symptomatic infection. This lack of discrimination conflicts with the results presented here, by Schafer et al (1993) (271) and by Mutimer et al (1995) (273) and may be due to 'saturation' of nested PCR reactions, leading to an underestimate of viral load.

3.4.6 PCR Positivity May Occur before the Onset of Symptoms

The timing of PCR positivity is crucial. In this study, qualitative PCR positivity of serum occurred before disease in 75% (6/8) patients; the quantitation 'threshold' was exceeded before disease in 57% (4/7) patients (and was exceeded simultaneously with disease for 43% (3/7) patients). These results suggest that these PCR tests will only enable pre-emptive prophylactic treatment for a proportion of patients but will nevertheless, provide useful confirmation of CMV infection for all patients thus facilitating treatment. However, samples were only taken twice weekly in this study; accurate study of timing should be conducted in a further study by testing serum samples on a daily basis. Other reports have shown that whole blood PCR positivity occurs before CMV disease in liver transplants (160, 227).

Finally, urine testing by PCR was not useful. Sensitivity, specificity and prediction of CMV disease episodes was poor (see also 160, 161, 162, 163, 164 and Chapter 2).

3.4.7 Active CMV Infection and Bone Marrow Transplantation

A small number of bone marrow transplant patients were included in this study; results must therefore be interpreted with caution. However, some interesting observations could be made.
All patients that developed symptomatic CMV infection were recipient CMV antibody positive/ donor negative combinations. Furthermore, all patients that developed active CMV infection also developed acute graft versus host disease.

Active cytomegalovirus infection and graft versus host disease have been linked (see section 1.6.6).

**The Effect of CMV on GVHD**

A number of papers have suggested that CMV infection may predispose patients to acute (131) or chronic GVHD (132, 133, 134, 135). Lonqvist 1984 (132) studied 53 allograft recipients surviving more than three months; the incidence of chronic GVHD was 42% overall and was higher after CMV disease (56% (20/36) versus 12% (2/17); p<0.01). CMV disease occurred before GVHD by a median of 128 days (range 23-322 days). Other herpesviruses have also been implicated (136, 137).

Donor CMV antibody positivity and not active CMV infection was shown to be important by Jacobsen et al 1986 (134). Donor immunity to CMV is implicated and the authors suggest that CMV stimulation of lymphokines or MHC restricted presentation of CMV peptides mediates GVHD. CMV specific cytotoxic T-lymphocyte (CTL) activity of patients with active CMV infection is higher in those whose donor is CMV immune because it is the donor immune system that reconstitutes after allogeneic bone marrow transplantation (138).

Acute GVHD is also a risk factor for chronic GVHD; it is possible that epidemiological associations between chronic GVHD and CMV infection result because CMV activation is a marker of acute GVHD.
The Effect of GVHD upon Active CMV Infection and CMV Pneumonitis

However, it is also possible that graft versus host disease predisposes to active CMV infection.

CMV has been found within skin and gut tissue during episodes of acute GVHD (139, 140). However, when Appleton et al 1995 analysed sequential rectal biopsies for acute GVHD and CMV infection it was found that GVHD preceded active CMV. Other authors also suggest that it is GVHD that initiates or amplifies active CMV infection (141, 142, 143, 139).

Meyers et al 1986 (141) studied 545 marrow recipients and found that the incidence of acute GVHD was not influenced by CMV serology and was 39% amongst CMV antibody negative recipient/negative donor combinations. Conversely, the incidence of active CMV infection, detected by buffy coat culture, was higher in recipients developing severe (grade II-IV) acute GVHD (63.1% versus 42.1%; p=0.0001). CMV infection occurred after acute GVHD developed (median 19 days). In addition, severe acute GVHD was a risk factor for the development of CMV pneumonia (p<0.0005).

Bowden et al (1987) (144) suggested that recipient CMV reactivation was initiated after GVHD-induced obliteration of recipient lymphocytes; natural killer cell responses to CMV-infected and uninfected cells were significantly reduced during GVHD.

In addition, acute GVHD is a risk factor for the development of CMV pneumonitis (141, 145, 148, 147, 143). It has been hypothesised that alloreactive lymphocytes are involved and that CMV modifies the
In addition, acute GVHD is a risk factor for the development of CMV pneumonitis (141, 145, 148, 147, 143). It has been hypothesised that alloreactive lymphocytes are involved and that CMV modifies the antigenic status of lung interstitial cells; Zaia 1986 (148) showed that the amount of CMV DNA present in lung tissue does not predict the severity of pneumonitis.

This is difficult to reconcile with a relatively small number of reports that suggest that T-lymphocyte depletion prior to bone marrow transplantation increases the risk of developing CMV pneumonitis despite lowering the incidence of acute GVHD (150, 151, 152). CMV-specific cellular responses are important after bone marrow transplantation (144, 153) and Hertenstein et al (1995) (150) suggest that T-cell depletion prevents an early and important cellular response to CMV thus allowing uncontrolled replication which predisposes to CMV pneumonitis which may, or may not, involve immune-mediated injury.

**Murine Models of GVHD and CMV Pneumonitis**

Grundy et al 1985 (154) injected immunocompetent (B10 x B10.A) F1 hybrid mice with parental spleen cells; GVH-induced immunosuppression of the recipient (F1 hybrid) immune system was measured by chromium release assay (CRA) after spleen cell cytolysis of allogeneic and trinitrophenol (TNP)-modified syngeneic target cells. Twenty times less parental spleen cells were needed to ablate the recipient immune system when murine cytomegalovirus (mCMV) was injected concomitantly. Only mice injected with parental spleen cells and mCMV died from CMV pneumonia; CMV was cultured from the lungs and inflammation was seen. This group went on to show that 87% of the infiltrating cells were donor specific (155). The authors suggest that the
observed synergy between mCMV and induced GVH was mediated by the, previously observed, ability of mCMV to increase alloreactivity (51; see section 1.9.3).

Ganciclovir significantly reduced the amount of mCMV isolated from the lungs and the degree of interstitial inflammation but did not reduce the frequency of pneumonitis nor the degree of perivascular inflammation (156). This paper emphasised the immunopathology of CMV pneumonitis and suggests that an alloreactive, rather than a mCMV-specific, cellular response is important.

This proposal is supported indirectly by the case reported in this thesis that died of CMV pneumonitis. Regular monitoring by serum and urine PCR showed that this case cleared CMV from both sites several days before death. Unfortunately, lung tissue from this patient was not available.
CHAPTER 4

RISK FACTORS FOR CHRONIC REJECTION OF LIVER GRAFTS INCLUDING CYTOMEGALOVIRUS
4.1 Introduction

A number of groups have attempted to elucidate the risk factors for chronic rejection after liver transplantation\textsuperscript{(204, 205, 210, 225, 283, 372)}; the importance of HLA matching/ mismatching, primary disease and cytomegalovirus (CMV) remain controversial (see section 1.8.2). A summary of these findings for CMV is given in table 4.1 and important points are described below.

**TABLE 4.1 Summary of Studies of CMV as a Risk Factor for Chronic Rejection of Liver Grafts**

<table>
<thead>
<tr>
<th>Group</th>
<th>Reference</th>
<th>CMV Risk</th>
<th>CMV Risk Identified</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kings College</td>
<td>O'Grady et al (1988) (283)</td>
<td>Yes</td>
<td>Active CMV infection</td>
<td>7.5</td>
</tr>
<tr>
<td>Kings College</td>
<td>Arnold et al (1992) (284)</td>
<td>Yes</td>
<td>Persistent graft infection</td>
<td>5.4</td>
</tr>
<tr>
<td>Pittsburgh</td>
<td>Manez et al (1993) (225)</td>
<td>Yes</td>
<td>CMV hepatitis</td>
<td>6</td>
</tr>
<tr>
<td>Birmingham</td>
<td>Candinas et al (1995) (210)</td>
<td>Yes</td>
<td>Serology mismatch (donor +/- recipient -)</td>
<td>3.5</td>
</tr>
<tr>
<td>Cambridge</td>
<td>P.E. (1996) (THESIS)</td>
<td>Yes</td>
<td>Active CMV infection for ≥ 1 month</td>
<td>3.3</td>
</tr>
<tr>
<td>Mayo Clinic</td>
<td>Paya (1992) (372)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These differences may have arisen because of different patient management regimes between centres such as the nature and degree of immunosuppression and the promptness of antiviral treatment.

Only one study \(372\) did not find a link between CMV and chronic rejection; here, all patients received acyclovir prophylaxis and were treated...
with ganciclovir after development of active CMV infection. However, CMV testing (by DEAFF and culture) was only performed when active CMV was suspected clinically; sub-clinical active infection was not analysed in this study and may have been important.

In this chapter, the involvement of CMV was analysed in detail; viral load and serum PCR positivity were shown to be important prognostic indicators of CMV disease in Chapter 3 and are now analysed in the context of chronic rejection. Other, previously published risk factors were also analysed for this group.

In addition, the role of the promoter allele TNF-2 was investigated in relation to chronic rejection and active CMV infection (see section 1.9.4); to my knowledge, this is the first study of its kind. This was undertaken because the TNF-2 promoter allele is associated with increased TNF expression (405) and elevated TNF levels have been described previously for chronically rejected livers (397). Furthermore, TNF expression and active CMV infection have been shown to positively upregulate each other (398, 399, 400, 401, 402; see section 1.9.4).

Hypotheses

- CMV infection may constitute a risk for chronic rejection of liver grafts.

- Class II MHC molecule matching may be a risk factor for chronic rejection.

- MHC molecule mismatching may be a risk factor for persistent active CMV infection.

- The tumour necrosis factor-2 (TNF-2) promoter allele, in liver transplant recipients, is a risk factor for chronic rejection and may interact with CMV.
4.2 Patients, Materials and Methods

4.2.1 Patients

Selection of Chronic Rejection Study Group and Controls

Initially, patients were selected according to the criteria that samples had been successfully collected post transplant for more than two months and at a rate of more than one sample (serum, whole blood and urine) per week (see section 2.2.6, see Appendix 1).

From this group, 33 patients were selected. All patients that were re-transplanted were selected (18 patients were re-transplanted; 12 of whom lost grafts to chronic rejection). In addition, 15 patients that did not lose their graft were selected to serve as controls; these were chosen sequentially according to transplant date. All patients studied had been transplanted within the past 3 years.

The 33 patients chosen for study received 57 allografts which were split into two groups; those that were lost to chronic rejection (n=12) and controls (n=45). The latter group comprised 18 primary transplants, 17 secondary transplants and seven tertiary transplants that were retained and three primary transplants that were lost (to reasons other than chronic rejection).

All of these post transplant courses were monitored prospectively for active CMV infection by serum and urine PCR, buffy coat and urine DEAFF and culture and/or serological change.
Selection of Patients for TNF Allele Study

The criteria for selection for this study was the availability of a pre-transplant whole blood sample; 123 liver transplant recipients (15 chronic rejectors and 108 controls) were tested for TNF allele status.

Data of active CMV infection was available for the majority of these patients (n=110). Of these, 33 patients were monitored by serum and urine PCR, buffy coat and urine DEAFF and culture and/or serological change whereas 77 patients were only monitored as part of routine clinical practice by buffy coat and urine DEAFF and culture and/or serological change. The latter group were therefore monitored using techniques of limited sensitivity and results from this group were gathered retrospectively.

4.2.2 Sample Collection

Sample collection, processing and storage was carried out according to the methods given in Chapter 2 (section 2.2.5).

4.2.3 Virological Data

Virological data (serology, DEAFF, serum PCR and urine PCR) was generated, without knowledge of clinical details, as described in Chapter 3.

4.2.4 Human Leukocyte Antigen (HLA) Allele Typing

HLA typing was performed by microcytotoxicity assay according to the method of Darke and Dyer (1992) (406) using a panel of sera with known HLA reactivity. Briefly, recipient and donor whole blood were collected in preservative free heparin before isolation of peripheral blood lymphocytes (PBL) on a Ficoll gradient. B-cells were enriched from a proportion of PBL after 'rosetting' of T-lymphocytes with sheep red blood
cells. PBL were used to determine HLA class I type and B cells to determine class II type. For each assay, cells were then counted and concentration was adjusted to $10^6$/ml before 1µl cells was added to each well of a microtitre plate containing a test sera. The plate was now incubated at 22°C for 30 minutes before 4µl of complement (rabbit serum) was added and a further incubation at 22°C for 30 minutes was performed. Staining was then carried out for five minutes using 2µl 5% aqueous eosin-Y before cells were fixed in formaldehyde. Plates were then centrifuged (200g/1 minute) to recover cells and cells were visualised by microscopy. Positivity was scored when cell death exceeded 20% total. Matching and mismatching of specificities was scored for each donor/recipient pair.

4.2.5 Tumour Necrosis Factor (TNF) Promoter Allele Typing
This was performed by PCR of a 184 bp region of the TNF-α gene promoter using sequence specific primers (408). Sense primers for amplification of TNF-1 or TNF-2 differed by a single, 3' terminal nucleotide at position -308 (if transcription start site is nucleotide +1); this single base mutation separates these 2 alleles. Two reactions were set up for each sample; PCR for the TNF-1 allele (reaction 1) incorporated antisense primer (5'-TCTCGGTTTCTTCTCCATCG-3'; nucleotides -164 to -144) and sense primer (5'-ATAGGTTTTGAGGGGCATG -3'; nucleotides -328 to -308). PCR for the TNF-2 allele (reaction 2) incorporated identical antisense primer but different, specific sense primer (5'-ATAGGTTTTGAGGGGCATGA -3'; nucleotides -328 to -308).

Primers for human growth hormone were included in each reaction as a positive control for successful amplification (342 bp PCR product). These comprised sense (5'-GCCTTCCCAACCATTCCCTA-3') and antisense (5'-TCACGGATTCTGTTGTTTT-3') primers.
Each reaction was set up in a final volume of 13µl containing 75mM tris-HCl (pH 9.0), 20mM (NH4)2 SO4, 0.01% Tween, 2mM MgCl2, 0.2µg template DNA, 200µM each dNTP, 10pmol each sequence specific primer, 2pmol each control primer, 0.15 Units Taq Polymerase. Thermal cycling was performed as follows: 10 cycles (94°C, 20 seconds/ 65°C, 60 seconds) followed by 20 cycles (94°C, 20 seconds/ 61°C, 50 seconds/ 72°C, 30 seconds).

Reaction mixes were then loaded and electrophoresed upon a 1.5% agarose gel (75V, 3 hours), stained with ethidium bromide and viewed over ultraviolet light. TNF typing was then scored for reactions that had undergone successful PCR amplification (ie. positive control product identified).

4.2.6 Clinical Data
Clinical details were gathered by two independent workers (Dr Jane Collier and Mr Avi Soin) without knowledge of virological details. CMV disease was defined by the parameters given earlier in Chapter 3. Acute rejection was defined by parameters given by Snover et al (1984) (192; see section 1.8.1.1) and chronic rejection was defined by the parameters set out by Wight D (1991) (95; also see section 1.8.1.2).

4.2.7 Results Analysis
Allografts were split into two groups; those lost to chronic rejection (n=12) and controls (n=45).

These two groups were analysed for CMV-related factors ie. serology, active infection, duration of active infection, time of initial positivity,
viral load and 'cumulative viral load'. Duration and initial positivity time were plotted for each patient studied and an attempt was made to draw an arbitrary threshold to discriminate chronic rejectors from controls. In addition, the thresholds drawn in Chapter 3 for viral load and 'cumulative viral load' are drawn here. The results shown in table 4.2 relate to these arbitrarily drawn thresholds.

In addition, factors that have previously been related to chronic rejection were analysed; i.e. primary disease (PSC, PBC, hepatitis C and hepatitis B virus infection), recipient and donor age and sex, recipient and donor HLA status (matching and mismatching of DR and A and B loci), incidence, number, timing and grade of acute rejection episodes.

Finally, the distribution of the TNF-2 allele among a larger group of consecutive liver transplant patients (15 chronic rejectors and 108 controls) was analysed.

Note that there is some variation in the number of grafts compared for different factors according to data availability.
4.3 Results

4.3.1 Cytomegalovirus as a Risk Factor for Chronic Rejection

4.3.1.1 CMV-Related Risk Factors for Chronic Rejection

These are shown in table 4.2a.

<table>
<thead>
<tr>
<th>TABLE 4.2a Cytomegalovirus as a Risk Factor for Chronic Rejection of Liver Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
</tr>
<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>CYTOMEGALOVIRUS</td>
</tr>
<tr>
<td>Serology</td>
</tr>
<tr>
<td>Donor - : recipient -</td>
</tr>
<tr>
<td>Donor - : recipient +</td>
</tr>
<tr>
<td>Donor + : recipient -</td>
</tr>
<tr>
<td>Donor + : recipient +</td>
</tr>
<tr>
<td>Active Infection</td>
</tr>
<tr>
<td>Incidence</td>
</tr>
<tr>
<td>Duration ≥ 30 days</td>
</tr>
<tr>
<td>Time initially positive ≥ 25 days</td>
</tr>
<tr>
<td>Serum PCR +</td>
</tr>
<tr>
<td>Incidence</td>
</tr>
<tr>
<td>Duration ≥ 30 days</td>
</tr>
<tr>
<td>Time initially positive ≥ 25 days</td>
</tr>
<tr>
<td>Peak viral load &gt; threshold</td>
</tr>
<tr>
<td>Cumulative viral load &gt; threshold</td>
</tr>
<tr>
<td>Urine PCR +</td>
</tr>
<tr>
<td>Incidence</td>
</tr>
<tr>
<td>Duration ≥ 30 days</td>
</tr>
<tr>
<td>Time initially positive ≥ 25 days</td>
</tr>
<tr>
<td>Symptomatic Infection</td>
</tr>
<tr>
<td>Incidence</td>
</tr>
<tr>
<td>Time initially positive ≥ 25 days</td>
</tr>
</tbody>
</table>

Table 4.2 shows the results from analysis of 57 liver transplants; 15 were chronically rejected and 42 served as controls. The following factors were compared between these 2 groups; cytomegalovirus (CMV) serology and active infection (incidence, onset, duration and load), serum and urine PCR positivity (incidence, onset, duration and load) and the incidence of symptomatic infection).

* Fishers exact test
4.3.1.2 Early Active CMV Infection after Retransplantation for Chronic Rejection

Indirect evidence that CMV persistence is associated with chronic rejection was sought by comparing the median time for the onset of active CMV infection after retransplantation between patients retransplanted for chronic rejection and controls. Results are given in table 4.2b, and important points are described below.

Table 4.2b Early Active CMV Infection Occurred after Retransplantation for Chronic Rejection

<table>
<thead>
<tr>
<th></th>
<th>Chronic Rejectors</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial Transplant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Transplants</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Active CMV</td>
<td>63%</td>
<td>59%</td>
</tr>
<tr>
<td>Onset (Median day post transplant)</td>
<td>44 (24-61)</td>
<td>41 (32-68)</td>
</tr>
<tr>
<td>% onset &lt; 25 days</td>
<td>20% (1/5) a</td>
<td>0% (0/10) b</td>
</tr>
<tr>
<td><strong>Retransplant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Transplants</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Active CMV</td>
<td>78%</td>
<td>67%</td>
</tr>
<tr>
<td>Onset (Median day post transplant)</td>
<td>10 (0-26)</td>
<td>36 (22-75)</td>
</tr>
<tr>
<td>% onset &lt; 25 days</td>
<td>86% (6/7) c</td>
<td>25% (1/4) d</td>
</tr>
</tbody>
</table>

Table 4.2b shows the median day after transplantation that active CMV infection occurred for initial transplants that were chronically rejected, initial transplants that were not chronically rejected, retransplants for chronic rejection and retransplants for other reasons. Of those patients that developed active CMV infection, a significantly higher proportion of patients that were retransplanted for chronic rejection had onset of active CMV infection <25 days posttransplant when compared to initial transplantation of these patients that developed chronic rejection (a versus c; p=0.06), initial transplantation of the control group (b versus c; p=0.00004) or retransplantation of the control group ie. grafts not lost to chronic rejection (c versus d; p=0.018). Significance was calculated using Fishers exact test.

Summary

- Donor negative/recipient positive CMV antibody status may be a risk factor for chronic rejection.
The incidence of 'prolonged' (30 days or more) active CMV infection or 'prolonged' urine PCR positivity was significantly greater for those that subsequently developed chronic rejection.

'Prolonged' active CMV infection may be a risk factor for chronic rejection.

Furthermore, the median onset of active CMV infection after chronic rejection was significantly earlier than the onset after primary transplantation (irrespective of graft outcome) or retransplantation for reasons other than chronic rejection.

4.3.2 Human Leukocyte Antigen (HLA) Alleles and Chronic Rejection

4.3.2.1 The Effect of HLA Matching/ Mismatching upon Chronic Rejection

The distribution of matched and mismatched HLA alleles is shown in table 4.3.

<table>
<thead>
<tr>
<th>HLA Status</th>
<th>CR Group (n=11)</th>
<th>Controls (n=33)</th>
<th>Relative Risk</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial or complete match</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA A Allele</td>
<td>45% (5/11)</td>
<td>55% (18/33)</td>
<td>0.83</td>
<td>0.4 - 1.7</td>
</tr>
<tr>
<td>HLA B Allele</td>
<td>27% (3/11)</td>
<td>27% (9/33)</td>
<td>1.0</td>
<td>0.32 - 3.05</td>
</tr>
<tr>
<td>HLA DR Allele</td>
<td>55% (6/11)</td>
<td>45% (15/33)</td>
<td>1.2</td>
<td>0.6 - 2.3</td>
</tr>
<tr>
<td>Complete mismatch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA A Allele</td>
<td>55% (6/11)</td>
<td>45% (15/33)</td>
<td>1.2</td>
<td>0.6 - 2.3</td>
</tr>
<tr>
<td>HLA B Allele</td>
<td>73% (8/11)</td>
<td>73% (24/33)</td>
<td>1.0</td>
<td>0.66 - 1.52</td>
</tr>
<tr>
<td>HLA DR Allele</td>
<td>45% (5/11)</td>
<td>55% (18/33)</td>
<td>0.83</td>
<td>0.4 - 1.7</td>
</tr>
</tbody>
</table>

Table 4.3 shows the proportion of patients that developed chronic rejection (CR group) and those that did not develop chronic rejection (controls) that were partially or completely matched or completely mismatched for HLA A, B and DR. The relative risk of developing chronic rejection is given with the 95% confidence interval.
Summary

- The proportion of matched and mismatched HLA-A, -B or -DR alleles did not vary between chronic rejectors and controls.

4.3.2.2 HLA Allele Combinations and Chronic Rejection

The distribution of HLA DR partial or complete matches/HLA A and B partial or complete matches and HLA DR partial or complete matches/HLA A and B complete mismatches is shown in Table 4.4.

Table 4.4 The Effect of HLA Allele Combinations on the Incidence of Chronic Rejection

<table>
<thead>
<tr>
<th>DR status</th>
<th>Class I status</th>
<th>CR group (n=15)</th>
<th>Controls (n=33)</th>
<th>Relative risk</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Match (≥1 allele)</td>
<td>HLA-A partial or complete mismatch</td>
<td>55% (6/11)</td>
<td>39% (13/33)</td>
<td>1.38</td>
<td>0.7-2.7</td>
</tr>
<tr>
<td>Match (≥1 allele)</td>
<td>HLA-B partial or complete mismatch</td>
<td>55% (6/11)</td>
<td>42% (14/33)</td>
<td>1.29</td>
<td>0.65-2.1</td>
</tr>
<tr>
<td>Match (≥1 allele)</td>
<td>HLA-A or B partial or complete mismatch</td>
<td>64% (7/11)</td>
<td>61% (20/33)</td>
<td>1.05</td>
<td>0.62-1.8</td>
</tr>
<tr>
<td>Match (≥1 allele)</td>
<td>HLA-A complete mismatch</td>
<td>36% (4/11)</td>
<td>21% (7/33)</td>
<td>1.70</td>
<td>0.6-4.8</td>
</tr>
<tr>
<td>Match (≥1 allele)</td>
<td>HLA-B complete mismatch</td>
<td>45% (5/11)</td>
<td>30% (10/33)</td>
<td>1.50</td>
<td>0.7-3.4</td>
</tr>
<tr>
<td>Match (≥1 allele)</td>
<td>HLA-A and B complete mismatch</td>
<td>45% (5/11)</td>
<td>36% (12/33)</td>
<td>1.25</td>
<td>0.6-2.8</td>
</tr>
</tbody>
</table>

Table 4.4 shows the proportion of patients that developed chronic rejection (CR group) and those that did not develop chronic rejection (controls) that were partially or completely matched or completely mismatched for HLA A, B and were partially or completely matched for HLA DR. The relative risk of developing chronic rejection is given with the 95% confidence interval (CI).

Summary

- The distribution of HLA DR partial or complete matching/HLA class I partial or complete matching combinations was similar for chronic rejectors and controls.

- The distribution of HLA DR partial or complete matching/HLA class I complete mismatching combinations was similar for chronic rejectors and controls.
4.3.3 The Effect of HLA Matching on the Duration of Active CMV Infection

The proportion of HLA class I and class II matched alleles were compared between patients hosting active CMV infection for 30 days or more and those actively infected for less than 30 days. This distribution is given in table 4.5.

Table 4.5

The Effect of HLA Allele Matching on the Duration of Active CMV Infection

<table>
<thead>
<tr>
<th>HLA status</th>
<th>Patients with Active CMV Infection</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duration &gt;30 days</td>
<td>Duration &lt;30 days</td>
</tr>
<tr>
<td>HLA-DR match (≥1 allele)</td>
<td>44% (4/9)</td>
<td>54% (7/13)</td>
</tr>
<tr>
<td>HLA-A match (≥1 allele)</td>
<td>22% (2/9)</td>
<td>46% (6/13)</td>
</tr>
<tr>
<td>HLA-B match (≥1 allele)</td>
<td>0% (0/9)</td>
<td>46% (6/13)</td>
</tr>
<tr>
<td>Class I match (≥1 allele)</td>
<td>11% (2/9)</td>
<td>46% (12/26)</td>
</tr>
</tbody>
</table>

Table 4.4 shows the proportion of patients that developed active CMV infection for more than (>30 days or less than (<30 days in relation to partial or complete matching for HLA A, B or DR. Fishers exact test was used.

Summary

- HLA class I matching (HLA-A or HLA-B) was found in a significantly higher proportion of patients with active CMV infection persisting for less than 30 days
- This result was also significant for HLA-B matching alone; a similar trend was seen for HLA-A matching alone but this result was not significant.
4.3.4 Other Risk Factors for Chronic Rejection of Liver Grafts

Other, previously published risk factors were analysed; the distribution of these factors is given in table 4.6.

**TABLE 4.6 Other, Possible Risk Factors for Chronic Rejection of Liver Grafts**

<table>
<thead>
<tr>
<th>Factor</th>
<th>CR group (n=12)</th>
<th>Controls (n=45)</th>
<th>Relative risk</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pretransplant disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic rejection</td>
<td>8% (1/12)</td>
<td>20% (8/40)</td>
<td>0.4</td>
<td>5.8x10^-2 - 3.0</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis (PSC)</td>
<td>8% (1/12)</td>
<td>2.5% (1/40)</td>
<td>3.3</td>
<td>0.22 - 49</td>
</tr>
<tr>
<td>Primary biliary cirrhosis (PBC)</td>
<td>25% (3/12)</td>
<td>2.5% (1/40)</td>
<td>10</td>
<td>1.14 - 87.5</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>33% (4/12)</td>
<td>12.5% (5/40)</td>
<td>2.67</td>
<td>0.8 - 8</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>8% (1/12)</td>
<td>0% (0/40)</td>
<td>3.7</td>
<td>p= 0.23 *</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient &gt; 30 years</td>
<td>100% (11/11)</td>
<td>95% (30/30)</td>
<td>1.05</td>
<td>0.24 - 2.43</td>
</tr>
<tr>
<td>Recipient &gt; 40 years</td>
<td>73% (8/11)</td>
<td>75% (30/40)</td>
<td>0.96</td>
<td>0.6 - 1.5</td>
</tr>
<tr>
<td>Recipient &gt; 50 years</td>
<td>64% (7/11)</td>
<td>55% (22/40)</td>
<td>1.15</td>
<td>0.7 - 1.96</td>
</tr>
<tr>
<td>Recipient &gt; 60 years</td>
<td>9% (1/11)</td>
<td>18% (7/40)</td>
<td>0.5</td>
<td>7x10^-2 - 3.8</td>
</tr>
<tr>
<td>Donor &gt; 30 years</td>
<td>40% (2/5)</td>
<td>65% (17/26)</td>
<td>0.6</td>
<td>0.2 - 1.9</td>
</tr>
<tr>
<td>Donor &gt; 40 years</td>
<td>20% (1/5)</td>
<td>41% (7/17)</td>
<td>0.41</td>
<td>0.7x10^-2 - 3.1</td>
</tr>
<tr>
<td>Donor &gt; 50 years</td>
<td>11% (1/7)</td>
<td>18% (3/17)</td>
<td>0.81</td>
<td>0.1 - 6.5</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male recipient</td>
<td>58% (7/12)</td>
<td>63% (26/41)</td>
<td>0.9</td>
<td>0.54 - 1.56</td>
</tr>
<tr>
<td>Male donor</td>
<td>50% (6/12)</td>
<td>44% (20/45)</td>
<td>1.25</td>
<td>0.6 - 21</td>
</tr>
<tr>
<td>Male recipient/ male donor</td>
<td>50% (6/12)</td>
<td>27% (12/44)</td>
<td>1.8</td>
<td>0.9 - 3.9</td>
</tr>
<tr>
<td>Male recipient/ female donor</td>
<td>8% (1/12)</td>
<td>34% (15/44)</td>
<td>0.24</td>
<td>3.5x10^-2 - 1.7</td>
</tr>
<tr>
<td>Female recipient/ male donor</td>
<td>0% (0/12)</td>
<td>18% (8/44)</td>
<td>0.43</td>
<td>p= 0.13 *</td>
</tr>
<tr>
<td>Female recipient/ female donor</td>
<td>42% (5/12)</td>
<td>20% (9/44)</td>
<td>2.0</td>
<td>0.8 - 4.9</td>
</tr>
<tr>
<td><strong>Acute Rejection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1 episode</td>
<td>58% (7/12)</td>
<td>47% (21/45)</td>
<td>1.25</td>
<td>0.7 - 2.2</td>
</tr>
<tr>
<td>≥2 episodes</td>
<td>42% (5/12)</td>
<td>2% (1/41)</td>
<td>17</td>
<td>2.2 - 132</td>
</tr>
<tr>
<td>Highest grade ≥2</td>
<td>42% (5/12)</td>
<td>34% (14/41)</td>
<td>1.22</td>
<td>0.6 - 2.7</td>
</tr>
</tbody>
</table>

Fifty patients were re-transplanted at Addenbrooke's NHS Trust over the past four years; 38% of these were re-transplanted after chronic rejection (see section 15.5; figure 1.4).

* Fishers exact test

**Summary**

- Two or more episodes of acute rejection conferred a significant risk for subsequent development of chronic rejection.
- Pre transplant diagnosis of primary biliary cirrhosis (PBC) conferred a significant risk for subsequent development of chronic rejection.
4.3.5 Cytomegalovirus and the TNF-2 Allele: Risk Factors for Chronic Rejection

4.3.5.1 The TNF-2 Allele and Chronic Rejection

The distribution of TNF genotypes with the incidence of chronic rejection is given in table 4.7.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Chronic Rejectors (n=15)</th>
<th>Controls (n=108)</th>
<th>Relative Risk</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-1 Homozygote</td>
<td>53% (8/15)</td>
<td>75% (81/108)</td>
<td>0.437</td>
<td>0.172 - 1.112</td>
</tr>
<tr>
<td>TNF-1/TNF-2 Heterozygote</td>
<td>40% (6/15)</td>
<td>22% (24/108)</td>
<td>2.067</td>
<td>0.801 - 5.33</td>
</tr>
<tr>
<td>TNF-2 Homozygote</td>
<td>7% (1/15)</td>
<td>3% (3/108)</td>
<td>2.125</td>
<td>0.363 - 12.45</td>
</tr>
<tr>
<td>TNF-2 Allele (homozygote or heterozygote)</td>
<td>47% (7/15)</td>
<td>25% (27/108)</td>
<td>2.29</td>
<td>0.9 - 5.831</td>
</tr>
</tbody>
</table>

TNF-2 promoter allele positivity (analysed as heterozygote genotype or allele positivity) in liver graft recipients was a risk factor for chronic rejection with borderline significance.
4.3.5.2 Active CMV Infection and the TNF-2 Promoter Allele May Synergise as Risk Factors for Chronic Rejection

The relative risk of chronic rejection from TNF-2 positivity was elevated in patients with active CMV infection in their post transplant course (see table 4.8).

<table>
<thead>
<tr>
<th>TNF Promoter Allele Status</th>
<th>CMV Status</th>
<th>Chronic Rejectors (n=14)</th>
<th>Controls (n=96)</th>
<th>Relative Risk</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-2 allele positive</td>
<td>Active CMV</td>
<td>36% (5/14)</td>
<td>13% (12/96)</td>
<td>3.039</td>
<td>1.16 - 7.965</td>
</tr>
<tr>
<td></td>
<td>No Active CMV</td>
<td>7% (1/14)</td>
<td>9% (9/96)</td>
<td>0.769</td>
<td>0.112 - 5.288</td>
</tr>
<tr>
<td>TNF-1 homozygous</td>
<td>Active CMV</td>
<td>43% (6/14)</td>
<td>42% (40/96)</td>
<td>1.043</td>
<td>0.388 - 2.804</td>
</tr>
<tr>
<td></td>
<td>No Active CMV</td>
<td>14% (2/14)</td>
<td>37% (35/96)</td>
<td>0.329</td>
<td>0.078 - 1.394</td>
</tr>
</tbody>
</table>

Table 4.8 shows the proportion of liver graft recipients that developed chronic rejection (chronic rejectors) and controls with the TNF-2 promoter allele (homozygotes and heterozygotes) and active CMV infection post transplant. The relative risk of developing chronic rejection and the 95% confidence interval (CI) are given.

Here, a greater proportion of patients that developed chronic rejection were positive for the TNF-2 promoter allele and active CMV infection when compared to controls. TNF-2 promoter allele positivity in combination with active CMV infection was a significant risk factor for chronic rejection.
4.3.5.3 Active CMV Infection and the TNF-2 Allele

The distribution of TNF genotype with the incidence of active CMV infection is given in table 4.9. TNF-2 positivity was distributed similarly between these two groups.

Table 4.9 Distribution of TNF-1 and TNF-2 Promoter Allele Genotypes with the Incidence of Active CMV Infection for 110 Liver Graft Recipients

<table>
<thead>
<tr>
<th></th>
<th>Active CMV Infection (n= 64)</th>
<th>Controls (n= 46)</th>
<th>Relative Risk</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-1 Homozygote</td>
<td>73% (47/64)</td>
<td>78% (36/46)</td>
<td>0.899</td>
<td>0.637 - 1.27</td>
</tr>
<tr>
<td>TNF-1/TNF-2 Heterozygote</td>
<td>20% (13/64)</td>
<td>22% (10/46)</td>
<td>0.964</td>
<td>0.647 - 1.438</td>
</tr>
<tr>
<td>TNF-2 Homozygote</td>
<td>6% (4/64)</td>
<td>0% (0/46)</td>
<td>p= 0.138*</td>
<td></td>
</tr>
<tr>
<td>TNF-2 Allele (homozygote or heterozygote)</td>
<td>27% (17/64)</td>
<td>22% (10/46)</td>
<td>1.112</td>
<td>0.787 - 1.57</td>
</tr>
</tbody>
</table>

* Fishers exact test

No significant differences were seen for the distribution of the TNF-2 allele between patients with active CMV infection and controls.

Summary

- TNF-2 promoter allele positivity (homozygote genotype or allele per se) was found in a significantly higher proportion of chronic rejectors compared to controls.

- Active CMV infection in combination with TNF-2 promoter allele positivity gave a higher significant relative risk for chronic rejection than TNF-2 promoter allele positivity alone. This suggested that these two factors act with synergy.

- TNF-2 promoter allele positivity was distributed similarly between patients with active CMV infection and controls.
4.4 DISCUSSION

Fifty six observations have been tested in this analysis of 33 liver transplant recipients (57 liver transplants) and it is possible that some observations have reached significance by chance. Therefore, the results of this broad analysis must be interpreted with caution. However, the results of interest set up hypotheses to be tested in a larger study.

Only three analyses of the TNF-2 allele were performed for over 100 liver transplant recipients and the results of this partly retrospective study may be interpreted more firmly.

4.4.1 Conclusions

Cytomegalovirus

- Donor negative/recipient positive CMV antibody status may be a risk factor for chronic rejection.
- Prolonged active CMV infection may be a risk factor for chronic rejection.

Human leukocyte antigens

- Matching or mismatching of HLA A, B or DR alleles are probably not risk factors for chronic rejection.
- HLA class II partial or complete matching in combination with HLA class I complete mismatching is probably not a risk factor for chronic rejection.
- HLA class I matching may be an important factor for prompt clearance of active CMV infection and may therefore be indirectly linked to chronic rejection.

Other Risk Factors

- Two or more episodes of acute rejection may be a risk factor for chronic rejection.
- Pre transplant diagnosis of PBC may be a risk factor for chronic rejection.
• Pre transplant diagnosis of PSC, hepatitis C or B or chronic rejection and recipient/donor age and sex are probably not risk factors for chronic rejection. However, a relatively small number of patients were analysed and a type II error may have occurred.

Tumour Necrosis Factor Alleles

• TNF-2 promoter allele positivity may be a risk factor for chronic rejection.

• Active CMV infection may synergise with TNF-2 promoter allele positivity as a risk factor for chronic rejection.

• TNF-2 promoter allele positivity is probably not an influence upon the incidence of active CMV infection.

4.4.2 Prolonged Active CMV Infection may be a Risk Factor for Chronic Rejection

Patients that developed chronic rejection and controls who did not develop chronic rejection were distributed according to the duration of active CMV infection that was experienced. From this distribution, an arbitrary threshold of 30 days active CMV infection was chosen; this threshold best separated these two groups (chronic rejectors and controls).

Active CMV infection for 30 or more days, diagnosed by serum PCR and/or urine PCR and/or urine DEAFF, constituted a risk for chronic rejection. This was seen when the duration of active CMV infection of urine was analysed separately but not after separate analysis of serum data. A similar trend was observed for the duration of active CMV infection in serum but this did not reach significance; this may be because low numbers were tested.

Active CMV infection and viral load of serum were shown to be important prognostic factors for acute symptomatic CMV infections (see
chapter 3). Active CMV infections were initiated at similar times for discrete periods and the duration of infection was not a prognostic indicator. This contrasts with the nature of CMV infections that were shown to be associated with chronic rejection; duration was important whereas the appearance of recognised symptoms and viral load were not.

Active CMV infection per se did not correlate with the incidence of chronic rejection. This contrasts with the report by O'Grady et al (1988) (283). However, urine PCR positivity was associated with chronic rejection. It is possible that an association between serum PCR positivity and chronic rejection did not reach significance through lack of numbers.

Prolonged CMV infection (more than 30 days) occurred in 5/6 actively infected chronic rejectors; of these, 3/5 infections did not clear and persisted in serum until graft loss. The mechanism of such persistence is unclear because it is likely that an immune response is mounted; 2/3 persistent infections were temporarily symptomatic.

The association between persistent CMV infection and chronic rejection may explain the novel finding of very early active CMV infection after retransplantation for chronic rejection (median six days post transplant (range 0-24 days)); this was significantly earlier than infection after primary transplantation (irrespective of whether the graft was chronically rejected or not) and retransplantation for reasons other than chronic rejection.

Three chronic rejectors did not clear active CMV between initial and secondary transplantation and were therefore positive on the day of retransplantation (day 0); chronic rejectors that did apparently clear active CMV (ie. were negative by laboratory tests) were positive on days 4-24 after
retransplantation. The 'incubation period' for CMV after transplantation is remarkably constant (114, 115) and it is likely that the latter cases did not clear but had persistent, sub-clinical CMV infection at an undetectable level (the sensitivity of the PCR test was 500 genomes/ml; see Chapter 2). It is possible that such a viral load, undetectable yet greater than that of latent infection, was able to replicate to detectable levels relatively quickly after the immunosuppression that was administered after retransplantation.

These are novel findings but are supported by those of the Kings College group. Arnold et al (1992) (284) used *in situ* hybridisation to screen serial liver biopsies taken from patients with chronic rejection or uncomplicated CMV hepatitis. CMV DNA was found in the hepatocytes of all patients with CMV hepatitis and 10/12 of those with chronic rejection. The latter group were persistently CMV positive until the graft was lost whereas the CMV hepatitis group cleared CMV from hepatocytes (p<0.0005). Importantly, the principal sites of damage (large bile ducts and hepatic arteries; see section 1.9.1 ) were not examined. However, CMV infection of hepatocytes may have indirectly caused or promoted damage of these tissues by mediating local, clonal expansion of allospecific T-lymphocytes (see section 1.10.3) or, as suggested by the authors, by affecting the expression of inflammatory molecules (see section 1.10.4). The finding of persistence and the link with Arnold et al (1992) (284) inspired the *in situ* hybridisation work that is presented in Chapter 6.

Persistent CMV infection is an intriguing phenomenon but does not necessarily mediate or modify the process of chronic rejection (see section 1.9.7). Indeed, the rejection process may facilitate persistence. Sinclair and colleagues (48, 49) showed that monocytes are a site for latent CMV and
that differentiation into macrophages renders these cells fully permissive to active CMV infection. Foam cells in arteries are macrophages and are characteristic of chronic rejection (see section 1.8.1.2); it is possible that these cells import latent CMV to the graft and then facilitate CMV replication after differentiation. During active infection, CMV is also tropic for neutrophils; these cells may also import CMV to the chronically rejected graft as they infiltrate.

CMV replication may also be facilitated by smooth muscle cell proliferation after ischaemic injury; smooth muscle cells have been shown to be fully permissive to CMV in vitro. Finally, the increased surface expression of HLA molecules during chronic rejection may facilitate CMV entry, into rejection site cells, if the HLA molecule is a receptor for CMV, as postulated by McKeating et al (1987) (62).

4.4.3 CMV Antibody Status and Chronic Rejection

Recipient CMV antibody positivity in combination with donor CMV antibody negativity was shown to confer a significant risk for chronic rejection. This result is consistent with the proposal that persistent, low level replication of CMV was associated with chronic rejection.

It is possible that CMV antibody positive recipients that received grafts from CMV antibody negative donors were able to mount a cellular immune response to CMV. Such patients may therefore not develop symptomatic CMV infection and not be treated with antiviral drugs.

It is conceivable that such patients are able to mount an immune response that is sufficient to prevent symptomatic infection but is not sufficient to prevent asymptomatic, active CMV infection. Such patients may
therefore host active CMV infection for a prolonged period of time (see above).

Conversely, CMV antibody negative recipients of liver grafts from CMV antibody positive donors are unable to mount a cellular immune response to CMV and are therefore more likely to develop symptomatic infection; the results presented in Chapter 3 show that active CMV was detected in all of the mismatched patients studied. Patients with mismatched CMV antibody status were therefore more likely to be treated with ganciclovir and therefore not host prolonged active CMV infection which may be an important risk factor for chronic rejection (see above).

4.4.4 The Effect of HLA Matching/ Mismatching upon the Incidence of Chronic Rejection

There was no overall correlation between matching or mismatching of class I or class II HLA molecules with the incidence of chronic rejection; in addition, the combination of class II partial or complete matching with class I complete mismatching (as reported by the Kings College group (243, 283)) was not shown to be a risk factor for chronic rejection.

However, complete mismatching of class I alleles was shown to be associated with active CMV infections of long duration (≥ 30 days) which in turn, was shown to be associated with the incidence of chronic rejection. One plausible hypothesis is that persistent CMV infection of the graft is a risk factor for chronic rejection and that complete mismatching of class I alleles prevents successful cellular immune surveillance therefore facilitating such persistence. This hypothesis predicts that class I mismatching will only be important when a recipient is at risk of active CMV infection and may explain the lack of a direct association between
HLA class I mismatching and chronic rejection here and the positive association shown in larger studies (225, 243, 283). Certainly, the importance of class I/CD8+ T-lymphocyte surveillance in clearing active CMV infection has been shown after transplantation (65; see section 1.4.1.1).

The Kings Group (243, 283) also showed that class II matching is important and that this risk factor synergises with the risk contributed by active CMV infection; this has been supported by the Pittsburgh group (225) who show that DR matching is associated with the incidence of CMV hepatitis. Both groups suggest that class II presentation of CMV peptides is important.

HLA DR matching was not shown to be a risk factor for chronic rejection in this thesis but it is possible that this mechanism was not identified because of the low numbers studied.

Generally, class I alleles present peptides from endogenously produced proteins whereas class II present those from exogenously produced proteins (242). A graft with class I complete mismatch/class II partial or complete match cannot present viral proteins produced endogenously, due to lack of MHC restriction, and so CMV transcription/translation and viral replication may occur undetected and lead to lytic infection. Only after reinfection of the graft with these progeny virions can cellular immune surveillance be performed successfully by class II presentation of exogenously derived CMV peptides; the immunopathological destruction suggested by the Kings College and Pittsburgh groups may now occur.
Thus, mismatched class I may provide an important window in which CMV can replicate to numbers sufficient to cause graft damage upon subsequent class II/ T-lymphocyte surveillance.

4.4.5 Recipients with the TNF-2 Promoter Allele May be at a Greater Risk of Developing Chronic Rejection

The link between TNF-2 promoter allele positivity and chronic rejection and its synergy with active CMV infection is intriguing.

The finding in this thesis of an association between recipient TNF-2 promoter allele positivity and chronic rejection is supported by Hoffman et al (1993) (397) who showed an association between infiltration by TNF expressing cells and chronic rejection. Here, chronic rejection of liver grafts was accompanied by portal tract and central venous infiltration of TNF-expressing macrophages and monocytes (identified by immunohistochemistry). Expression of the adhesion molecule ICAM-1 was elevated in hepatocytes adjacent to TNF-expressing macrophages and monocytes and in vascular endothelial cells. The authors proposed that infiltration of TNF-expressing cells led to further leukocyte infiltration after TNF-induced elevation of adhesion molecule expression upon vascular endothelial cells.

These results are intriguing because CMV persists latently in monocytes and reactivates upon their differentiation into macrophages (41, 48). Therefore, the data published by Hoffman et al (1993) (397) suggested that, for CMV antibody positive recipients, chronic rejection was associated with infiltration of actively infected cells.
Furthermore, there is evidence to suggest that active CMV infection is induced by TNF (398, 399). Docke et al (1994) (398) studied 60 patients that were receiving intensive care for various reasons (18 after abdominal surgery, eight after cardiovascular surgery, six after surgery to remove tumours (non-gastrointestinal), five after diagnosis of necrosing pancreatitis, three after diagnosis of liver cirrhosis and 20 for other reasons). Patients that developed sepsis were more likely to have detectable levels of TNF in plasma by immunoassay (61% versus 0%) and active CMV infection by PCR of peripheral blood mononuclear cells (PBMC) (92% versus 7%; p<0.001) compared to patients that did not develop sepsis.

This group (399) also studied liver and renal transplant recipients (n=190); episodes of acute rejection were significantly associated with elevated serum levels of TNF compared to non-rejecting controls (79% (60/76) versus 4% (1/25); p<0.01). Furthermore, correlation was found between elevated serum TNF and subsequent detection of CMV antigens in PBMC (88% >100pg/ml versus 16% <100pg/ml; p<0.001). The authors suggested that TNF enhanced CMV activity and also showed in vitro that TNF induced the CMV immediate-early promoter/enhancer using a chloramphenicol acetyl transferase (CAT) reporter construct transfected into the monocytic cell line HL-60. This paper therefore provides evidence for the 'alternative hypothesis' that CMV is an opportunist that is favoured by the processes of chronic rejection.

However, the results presented in this thesis showed that recipients with the TNF-2 promoter allele were not at higher risk of active CMV infection. It is possible that CMV is refractile to 'TNF-2 promoter allele-enhanced levels' of TNF.
In contrast, the clinical findings of the reports discussed may be due to induction or enhancement of TNF expression by CMV (400, 401, 402). Geist et al (1994) (402) found that active CMV infection upregulated TNF production by differentiated THP-1 cells (monocyte-derived macrophages); this in turn, was evidence that CMV may initiate or enhance the process of chronic rejection.

The effect of TNF upon CMV activity and the converse are not mutually exclusive and it is possible that a 'vicious circle' exists during the involvement of CMV with chronic rejection of liver grafts.

Finally, an interesting analogy exists between the involvement of TNF with chronic rejection and its involvement with cerebral malaria (403). Binding of the parasite Plasmodium falciparum to vascular endothelial cell adhesion molecules is crucial to the pathogenesis of this disease. Serum-TNF levels have been shown to correlate with the severity of cerebral malaria (403) and TNF has been shown to upregulate adhesion molecule expression (404). Binding of lymphocytes to vascular endothelial cells, before infiltration of liver grafts during chronic rejection, is analogous.

4.4.6 Recurrent Acute Rejection May be a Risk Factor for Chronic Rejection

The occurrence of two or more episodes of acute rejection was found to constitute a high risk for chronic rejection. This result is supported by previous findings (368, 285). Dousset et al (1993) (368) found that recurrent acute rejection and severity were risk factors for chronic rejection for 129 liver transplant recipients and that the timing of the rejection episodes
was not important. In this thesis, the discrepant finding of a lack of association with acute rejection severity may be due to low numbers; only eight cases of severe acute rejection (≥ grade 3) were present in this analysis.

Recently, the Birmingham group have reported that the occurrence of one or more episodes of acute rejection correlated with the incidence of chronic rejection. This report (210) may be misleading (Avi Soin; personal communication) because single episodes of acute rejection (previously reported to be unlinked to chronic rejection; 285, 368) were pooled with recurrent episodes (previously reported to constitute a significant risk of chronic rejection; 285, 368).

Acute and chronic rejection have distinct pathologies (see section 1.8.1) and the former is not a prerequisite for the latter (8/15 patients in this analysis experienced chronic, but not acute, rejection); therefore, the mechanism accounting for this link is intriguing. Soin et al (1995) (285) suggest that chronic rejection can be split into two categories based on the presence or absence of cellular infiltrates and that ductopenic rejection is secondary to ischaemic injury (mediated by humoral immunity) in the latter condition. They suggest that the former condition, mediated by cellular infiltration, develops from acute rejection.

It is possible that acute rejection mediates a 'non-specific' inflammatory response involving upregulation of HLA molecules, adhesion molecule and cytokines (369, 370, 371).
4.4.7 Pretransplant Diagnosis of Primary Biliary Cirrhosis (PBC) may be a Risk Factor for Chronic Rejection

Primary diagnosis of PBC was shown to confer a significant risk for chronic rejection; this result is supported by the findings of others (200, 201, 202, 210).

PBC is an autoimmune diseases that is characterised by the presence of circulating autoantibodies (see section 1.9.5.3). However, a causal relationship has not been established.

Furthermore, a number of reports suggest that the humoral response is an important mediator of chronic rejection (293, 294, 286; see section 1.8.2.4) and it is possible that the antibodies associated with PBC are involved. These reports inspired an in-depth study of the effect of these and other antibodies upon chronic rejection which is presented in Chapter 5.

Markus et al (1988) (240) suggested an intriguing hypothesis linking HLA status and autoimmune disease. Here, 507 liver allograft recipients were studied and it was found that, although HLA-DR matching was associated with reduced graft survival (p=0.054), mismatching of this locus was associated with chronic rejection (p=0.007). The authors suggest that DR matching facilitates the recurrence of autoimmune diseases such as PBC and primary sclerosing cholangitis (PSC).

In contrast, although pre transplant diagnosis of PSC was shown to confer a relative risk for chronic rejection of 1.82, this result was not significant. PSC is also an autoimmune diseases that is characterised by the presence of circulating autoantibodies (see section 1.9.5.3) but a causal relationship has not been established. The lack of a significant association between PSC and
chronic rejection that is reported in this thesis may be due to the relatively low number of patients studied; larger studies have found significant associations of chronic rejection with pre transplant diagnosis of PSC (203, 205, 206, 372).

4.4.8 Lack of Correlation Between Chronic Rejection and Sex, Age or CMV Antibody Mismatch

Sex mismatch (male donor/ female recipient), CMV antibody mismatch (donor +/ recipient -) and recipient age < 30 years were positively associated with chronic rejection by the Birmingham group (210). These parameters were not shown to be important in this study, possibly because of low numbers (type II error).

4.4.9 Retransplantation was Not Shown to be a Risk Factor for Chronic Rejection

In this analysis, 24/57 liver transplants were retransplants; 12/24 were retransplanted for chronic rejection. Previous study at Addenbrooke's NHS Trust (Soin A; unpublished results) and other centres (315, 322, 353) showed that retransplantation for chronic rejection conferred a high risk for recurrence of this disease. Study of chronic rejection after retransplantation may introduce bias but this trend was not seen in the highly selected group that was studied in this thesis and may be reversed (7% chronic rejectors and 29% controls had previous chronic rejection).
CHAPTER 5

THE RELATIONSHIP BETWEEN CHRONIC REJECTION OF LIVER GRAFTS, ACTIVE CYTOMEGALOVIRUS INFECTION AND HUMORAL IMMUNE RESPONSES TO BILE DUCT AND HEPATIC ARTERY
5.1 Introduction

Few studies have focussed on the role of humoral immunity during chronic rejection of liver grafts; a number of groups have studied the effect of lymphocytotoxic antibodies in this setting but these findings are conflicting (see section 1.8.2.4).

This chapter presents an analysis of antibodies to hepatic artery and bile duct and their relationship with chronic rejection of liver grafts; to my knowledge, such work has not been previously reported. However, a link between antibodies and chronic rejection of renal (305-311; see section 1.8.3.3.3) and cardiac (309, 311; see section 1.8.3.3.3) grafts has been previously reported.

Active CMV infection is common after transplantation (see Chapter 3) and has been shown to be associated with chronic rejection (225, 283; see Chapter 4). A number of mechanisms have been proposed for this association including modulation of antibody production (see section 1.9.5); active CMV infection has previously been shown to be associated with autoantibody production (257, 310, 312, 313, 314). In addition, it has been shown previously that CMV encodes a protein (H301 gene) with homology to HLA class I and that antibodies to this CMV protein are cross-reactive with HLA class I (60). In this chapter, antibodies to hepatic artery and bile duct were analysed in relation to active CMV infection.

Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are diseases that are characterised by the presence of a number of autoantibodies (317, 320; see section 1.9.6) and have also been associated
with an increased risk of chronic rejection (197, 210, 203, 205, 206; see section 1.8.2.5). In this thesis, pretransplant diagnosis of PBC was also shown to be a risk factor for chronic rejection (see Chapter 4). In this chapter, antibodies to hepatic artery and bile duct were analysed in relation to pre-transplant diagnosis of PBC and PSC.

In this chapter, the presence of serum antibodies to bile duct and hepatic artery proteins was compared between chronic rejectors and controls, patients that developed active CMV infection and controls and patients with pretransplant diagnosis of autoimmune disease and controls.

Hypotheses

- Generation of antibodies to hepatic artery and bile duct tissue may initiate or enhance chronic rejection.

- Active cytomegalovirus (CMV) infection of hepatic artery or bile duct may upregulate the expression of target antigens.

- Active CMV infection may upregulate the production of antibodies involved in chronic rejection.

- Antibodies produced to CMV may cross react with cellular antigens involved in chronic rejection.

- Autoantibodies associated with primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) are present after transplantation and may be associated with chronic rejection.
5.2 Patients, Materials and Methods

This study was split into two phases; phase I involved screening of sera after Western blotting of a variety of tissues. This phase was labour intensive and so a small number of sera were screened. Trends identified in phase I were assessed further in phase II where further sera were screened after Western blotting a restricted panel of tissue; where putative target antigens had been identified, the results of phase I and phase II were combined for statistical analysis.

5.2.1 Selection of 38 Liver Transplant Recipients

Ninety nine patients were transplanted during the course of this thesis; patients were initially selected according to the criteria that appropriate serum was available for this study (pre-transplant and 3 months post-transplant). A highly selected group of 38 patients undergoing 43 liver transplants was chosen from this subgroup and tested during this study; all re-transplanted patients were selected plus other patients of interest ie. those with active CMV infection and those with a pretransplant diagnosis of PBC or PSC (taken sequentially from sub-group according to date of transplant). This group is described briefly in table 5.1 (see also Appendix 1).

Sera from 17 patients undergoing 20 liver transplants were screened during phase I with selection biased towards patients that developed chronic rejection and/or active CMV infection (see table 5.1 and Appendix 1).
Table 5.1 Patients Studied in Chapter 5

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<th>Patient number</th>
<th>Transplant number</th>
<th>CR/ Redo</th>
<th>Active CMV (Y/N)</th>
<th>PBC/PSC</th>
<th>Serum used Pre</th>
<th>Post</th>
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Table 5.1 shows the patients (n=38) and transplants (n=43) studied in Chapter 5. In this table, CR= patients that lost the liver graft to chronic rejection, Redo= patients that lost the liver graft for reasons other than chronic rejection. This table also shows patients that developed active CMV infection (Y= yes, N= no), those with pre transplant diagnosis of primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC). Serum used indicates which pre and post transplant sera were studied; transplants shown in bold indicate those studied in phase I of this chapter. These patients are described further in Appendix 1.
5.2.2 Western Blotting

5.2.2.1 Protein Extraction and Quantitation
Tissue samples (1-5 g) were cut into fragments using a scalpel before suspension in 2 ml homogenisation buffer (0.25 M sucrose (Gibco), 15mM Tris (Gibco), 0.1mM EDTA (Sigma); pH 6.8) and homogenisation using a mortar and pestle containing liquid Nitrogen. The sample was then transferred to a 2ml microfuge tube and stored at -20°C.

Bovine serum albumin (BSA) was diluted to attain concentrations of 5, 10, 15, 20, 25 μg/ml and used to generate a standard curve for protein quantitation. Extracted tissue samples and 'BSA standards' were pipetted into wells of a microtitre plate and adjusted to 80μl before addition of 20μl BioRad Protein Assay Concentrate Solution and incubation at 37 °C for 1 hour. Optical density (OD620nm) was read using a Flow Titerterk Multiplate Washer (ICN-Flow) and a standard curve was produced enabling the concentration of test samples to be calculated.

5.2.2.2 SDS-Polyacrylamide Gel Electrophoresis and Blotting
SDS-polyacrylamide gels were formed using a BioRad Mini-Gel Apparatus. The 'resolving gel' (10% acrylamide (stock: 30% acrylamide, 0.3% bis-acrylamide; Sigma), 0.375M Tris (stock: 1.5M Tris (pH 8.8); Gibco), 0.1% SDS (Sigma), 0.1% ammonium persulphate (APS; Sigma), 0.1% (v/v) Temed (added last; Sigma)) was formed before formation of 2cm of 'stacking gel' (6% acrylamide, 0.125M Tris (stock: 0.5M Tris (pH 6.8)), 0.1% SDS, 0.1% APS, 0.1% Temed)).

A total of 10 μg of protein was loaded onto each lane and electrophoresed in 'running buffer' (2.8% glycine (Sigma), 0.6% Tris (Gibco), 0.1% SDS
(Sigma)) at 75 V for 2 hours following Laemmli UK et al (1970) (376). Proteins were thus separated by charge. Transfer was made onto Immobilon P Membrane (Millipore) by semi-dry Western blotting at 18V for 4 hours in 'transfer buffer' (0.58% Tris (Gibco), 0.29% glycine (Sigma), 0.04% SDS (Sigma), 20% (v/v) methanol (BDH)).

5.2.2.3 Detection of Serum Antibodies
Membranes were blocked (2.5% casein (Sigma), 0.9% NaCl (Gibco), 0.12% Tris (Gibco), 0.02% thiomersal (Sigma); pH 7.6) for 18 hours at room temperature with mixing before application of sample sera at a concentration of 1% (v/v) in 'wash buffer' (0.9% NaCl (Gibco), 0.5% casein (Sigma), 0.12% Tris (Gibco), 0.02% thiomersal (Sigma); pH 7.6) and incubation, with mixing, at 4°C for 18 hours.

Non-specifically bound antibody was removed by washing (at room temperature); this was performed sequentially in 'wash buffer' for 5 minutes, 'detergent buffer' ('wash buffer' with 0.5% Triton X-100 (Sigma), 0.1% SDS (Sigma)) for 5 minutes then twice in 'wash buffer' for 10 minutes.

Secondary antibody was then applied in 'wash buffer' and incubated at 4°C for 18 hours with mixing. Anti-human IgG (Sigma), anti-human IgA (Sigma) and anti-goat IgG (A-4062, Sigma) were used; all were alkaline phosphatase conjugated and were added at a dilution of 1:2000.

Non-specifically bound antibody was removed by 4 x 10 minute washes, at room temperature, with 'wash buffer' and then with tris buffered saline-tween (TBST; 1.17% NaCl (Gibco), 0.61% Tris (Gibco), 0.2% Tween-20 (BDH)).
Blots were then developed with 0.0165% NBT (stock: 50 mg/ml in 70% DMF; Sigma) and 0.033% BCIP (stock: 50 mg/ml in 100% DMF; Sigma) in 'developing buffer' (0.1M NaCl (Gibco), 0.1 M Tris (Gibco), 5mM MgCl$_2$ (Gibco); pH 9.5).

5.2.2.4 Investigation of Cross-Reactivity

In these experiments (see section 5.3.4.7), polyclonal antibodies to purified hexon protein from adenovirus type 2 (0151-9004; Biogenesis) or polyclonal antibodies to whole CMV virions (AD169 strain) (2470-5004; Biogenesis) were included in 'blocking buffer' during membrane blocking. All other steps were carried out as described above.

5.2.3 Phase I

Western blotting of a panel of various tissues was performed in Phase I of this study; this enabled a broad analysis to be carried out in an attempt to identify immunogenic tissues for study in phase 2 and to identify antigens that were upregulated by CMV infection and/or chronic rejection.

In addition, sera from patients of interest (see table 5.1) were screened in an attempt to identify antibodies to bile duct or hepatic artery tissue that were associated with chronic rejection, active CMV infection or pretransplant diagnosis of PBC.

Sera from two healthy individuals (CMV antibody positive and negative individuals) were tested against the panel of tissue as a further control.
IgA Antibodies to Bile Duct Tissue

The reactivity of IgA antibodies in pre- and post-transplant sera from 17 patients receiving 20 liver grafts (see table 5.1) to proteins of seven tissues/cells were investigated; these tissues/cells are listed below:

1. Bile duct from an explanted, grafted liver that was not lost to chronic rejection and was not infected by CMV ('transplanted' bile duct).

2. Bile duct from an explanted, grafted liver that was lost to chronic rejection but was not infected by CMV ('chronically rejected' bile duct).

3. Bile duct from an explanted, grafted liver that was lost to chronic rejection and was infected by CMV ('chronically rejected, CMV infected' bile duct).

4. Bile duct from an explanted liver from a patient that underwent primary transplantation for PSC ('PSC-diseased' bile duct).

5. Liver from an explanted liver from a patient that underwent primary transplantation for PBC ('PBC-diseased' liver).

6. CMV infected fibroblasts (containing $10^6$ pfu).

7. Uninfected fibroblasts.

Ideally, IgG, IgA and IgM antibodies would have been analysed. However, this work was labour intensive (bands were analysed for 440 Western blot lanes during phase 1 of this study) and therefore, only one isotype was investigated. I chose to investigate the presence of IgA antibodies against bile duct tissue because there is evidence to suggest that this isotype is predominantly produced when target antigens are expressed in bile duct/gut tissue (409, 418, 419, 420, 421).
IgG Antibodies to Hepatic Artery Tissue

The reactivity of IgG antibodies in pre- and post-transplant sera from 17 patients receiving 20 liver grafts (see table 5.1) to proteins of 4 tissues/cells were investigated; these tissues/cells are listed below:

1. Hepatic artery from an explanted, grafted liver that was not lost to chronic rejection and was not infected by CMV ('transplanted' hepatic artery)

2. Hepatic artery from an explanted, grafted liver that was lost to chronic rejection but was not infected by CMV ('chronically rejected' hepatic artery)

3. CMV infected fibroblasts (containing $10^6$ pfu)

4. Uninfected fibroblasts

Unfortunately, hepatic artery from a CMV-infected liver graft was not available. Ideally, IgG, IgA and IgM antibodies would have been analysed but practical constraints prevented this (see above). I chose to investigate the presence of IgG antibodies against hepatic artery tissue because there is evidence to suggest that this isotype is predominantly produced when target antigens are expressed in vascular tissue (410, 422).

Phase I Results Analysis

After Western blotting, the size of each band was identified and results for each serum/tissue combination were logged. A total of 280 and 160 lanes were analysed with respect to IgA antibodies and IgG antibodies respectively. Four rounds of univariate results analysis were performed to compare 'chronic rejection sera' (CR-sera), CMV sera, PBC-sera and PSC-sera with 'others'. The proportion of 'test sera' and 'other sera' that were positive for a particular band was plotted for each tissue tested; thus, 28 and 16 such graphs were analysed and compared with respect to IgA and
IgG antibodies respectively. An example graph is shown in figure 5.1; this plot shows the analysis of IgA antibodies in 'CR-sera' versus 'other sera' after Western blotting of 'chronically rejected, CMV infected bile duct'.

These analyses enabled investigation of the relation between chronic rejection, active CMV infection, PBC, PSC and antibody production pre- and post- transplant.

Furthermore, comparison of 44 such plots enabled investigation of the effect of chronic rejection and/or CMV infection on antigen expression; ie. comparison of 'chronically rejected, CMV infected', 'chronically rejected' and 'transplanted bile duct' and comparison of 'chronically rejected' and 'transplanted' hepatic artery. Plots for uninfected fibroblasts were made to investigate whether these antigens were specific to bile duct or hepatic artery and comparison with the plot for CMV-infected fibroblasts was made to investigate CMV-mediated effects on antigen expression.

'PBC liver' and 'PSC bile duct' were included as controls for PSC and PBC sera. It was thought that the reactivity of PBC- and PSC-associated antibodies may be pronounced in these tissues. In addition, these tissues were investigated for disease-specific antigens.
Figure 5.1  Example of a Plot Analysed in Phase I

Figure 5.1 shows the percentage of patients (y axis) that were positive for each antigen identified (size in kD shown numerically on x axis) for ‘chronically rejected/CMV infected’ bile duct tissue by pre transplant (PRE) and post transplant (POST) sera. In this particular analysis, patients were divided into those that lost the graft to chronic rejection (CR) and others (CONTROLS). The 39kD and 94kD antigens were selected for further study in phase II.
5.2.4 Phase II

IgA Antibodies

The trends observed in Phase I were studied further in Phase II by screening for IgA antibodies to the following tissue:

1. Bile duct from an explanted liver that was lost to chronic rejection and was infected by CMV (‘chronically rejected, CMV infected’ bile duct; see phase I)

2. Bile duct taken from a 'cut down' donor liver (‘normal’ bile duct)

IgG Antibodies

Screening for IgG antibodies to the following tissue was performed:

1. Hepatic artery from an explanted liver that was lost to chronic rejection but was not infected by CMV (‘chronically rejected’ hepatic artery; see phase I)

2. Hepatic artery taken from a 'cut down' donor liver (‘normal’ hepatic artery)

Chronically rejected hepatic artery and chronically rejected, CMV infected bile duct tissue were used to screen sera from further patients. In addition, normal hepatic artery and bile duct tissue were screened with all sera as a further control. For statistical analysis the results generated during phases I and II were combined. Therefore, the results presented in phase II were generated from screening all the sera shown in table 5.1; this sera was taken from 38 patients undergoing 43 transplants (described in table 5.1). Statistical analysis of results was carried out using Fishers exact test.

5.2.5 Crossreactivity Between Antibodies Generated to CMV and Cellular Proteins

Finally, an experiment was carried out to test antibodies of interest for cross reactivity with CMV.
5.3 RESULTS

5.3.1 Coomassie Stain of Western-blotted Tissue

Various tissues were used in stage I of this study. Firstly, coomassie staining was performed on Western blotted protein extractions from these tissues. Figure 5.2 shows that bile duct, hepatic artery, liver and cultured fibroblasts had differing protein contents which was manifest as differences in band number, size and intensity. Protein separation under these conditions of electrophoresis was good and loading was similar.

![Coomassie Stain of Western Blotted Proteins Extracted from Bile Duct, Hepatic Artery, Liver and Fibroblasts.]

Lane 1= molecular weight marker (Gibco 2µl), lane 2= PSC bile duct, lane 3= PBC liver, lane 4= uninfected fibroblasts, lane 5= CMV infected fibroblasts, lane 6= CMV negative/ non-CR/ transplanted bile duct, lane 7= CMV negative/ CR/ bile duct, lane 8= CMV positive/ CR/ bile duct, lane 9= non-CR/ transplanted hepatic artery, lane 10= CR hepatic artery.
5.3.2 Phase I: Analysis of Antibodies to a Panel of Various Tissues

Antigens identified by antisera were observed in a total of 440 lanes were analysed from four perspectives (see section 5.2.2) thus creating a mass of data; these data are summarised below.

5.3.2.1 Pretransplant and Posttransplant Sera Contained Numerous Antibodies to Bile Duct and Hepatic Artery Proteins

Pre- and post-transplant sera from liver transplant patients contained IgA which identified up to 18 antigens (36-175 kD) and IgG which identified up to 16 antigens (55-253 kD) in bile duct and hepatic artery respectively; example blots are shown in figures 5.3 (bile duct/ anti-IgA) and 5.4 (hepatic artery/ anti-IgG). All antigens were also found after screening fibroblast proteins.

5.3.2.2 Reactive Antibodies and Liver Disease

A relatively small number of antigens were recognised by antibodies present in the sera of two normal controls. Both sera contained IgA antibodies that reacted with antigens of 145, 134, 65 and 62 kD in all the bile duct tissues screened and IgG antibodies that reacted with antigens of 210, 185, 78 and 75 kD in all the hepatic artery tissues screened.

The majority of IgA and IgG antibodies were associated with liver disease and were not found in healthy individuals; this may have been due to enhanced exposure of antigens during liver disease.

However, none of the antigens identified by IgA or IgG antibodies were exclusive to patients with chronic rejection, active CMV infection, PBC or PSC suggesting that reactive antibodies were not specific to these conditions.
Figure 5.3
Examples of Western Blots of Bile Duct Tissue after Application of Transplant Patient Sera

Patient 1
Pre | Post
--- | ---
163 | 145
50

Patient 2
Pre | Post
--- | ---
163 | 145
94
85
80
65
62
39

Patient 3
Pre | Post
--- | ---
163 | 145
94
85
53
50
44
39

Figure 5.3 shows 3 examples of Western blots of bile duct proteins (chronic rejector/CMV positive). Patient 1 hosted primary, active CMV infection after transplantation and retained the graft; IgA with affinity to 3 protein bands appeared after transplantation. Patient 2 was retransplanted for reasons other than chronic rejection; IgA to 5 protein bands was seen pretransplant and to 10 bands posttransplant. Patient 3 lost the graft to chronic rejection; IgA to 7 protein bands was seen pretransplant and to 12 bands posttransplant. Band sizes are shown in kD.
Figure 5.4 shows three examples of Western blots of hepatic artery proteins from a graft that was lost to chronic rejection. Patient 1 lost the graft to chronic rejection; IgG to eight protein bands was seen pre transplant but to only five bands post transplant. Patient 2 hosted active CMV infection after transplantation and retained the graft; IgG with affinity to eight protein bands was seen pre transplant and to ten bands post transplant. Patient 3 did not host active CMV infection and retained the graft; IgG to nine protein bands was seen pre transplant and to nine bands post transplant (however, one band appeared and one band disappeared post transplant). Band sizes are shown in kilodaltons (kD).
5.3.2.3 IgA and Bile Duct Analysis

IgA Antibodies and Chronic Rejection

Two antigens were identified by antisera that discriminated between patients that developed chronic rejection (n=7) and controls (n=13; all others).

A 94kD antigen was found in a greater proportion of pre-transplant sera from chronic rejectors compared to controls for all bile duct tissue tested. This difference was pronounced for ‘chronically rejected, CMV infected’ bile duct; reactivity to the 94 kD antigen disappeared post-transplant (see figure 5.5). It is possible that this antibody conferred a risk for chronic rejection and that a 94 kD antigen was upregulated in bile duct by CMV or chronic rejection.

Figure 5.5 The Proportion of Sera from Patients that Developed Chronic Rejection (CR) and controls (NON-CR) that Contained IgA Antibodies to a 94kD Antigen in 'Chronically Rejected, CMV Infected Bile Duct'
Similarly, a 39kD antigen was discriminatory in bile duct tissues but more so in 'chronically rejected, CMV infected' bile duct (see figure 5.6). In contrast, reactivity to this antigen did not disappear after transplantation.

Figure 5.6 Proportion of Sera from Patients that Developed Chronic Rejection (CR) and controls (NON-CR) that Contained IgA Antibodies to a 39kD Antigen in 'Chronically Rejected, CMV infected Bile Duct'

IgA Antibodies and Active CMV Infection
One antigen of 44 kD discriminated between patients that developed active CMV infection (n=14) and controls (n=6; all others). This was only observed for 'chronically rejected/CMV infected' bile duct screened with post-transplant sera (see figure 5.7). It is possible that active CMV infection stimulated antibody production to a bile duct protein since this discrimination was not seen for CMV infected fibroblasts. However, a 44 kD band was recognised in fibroblasts by serum IgA from chronic rejectors and controls indicating that this antigen was not bile duct specific.

Figure 5.7 Proportion of Sera from Patients that Developed Active CMV Infection (CMV) and Controls that Contained IgA Antibodies to a 44kD Antigen in 'Chronically Rejected, CMV infected Bile Duct'
**IgA Antibodies and PBC and PSC**

No notable differences were seen between the antibodies detected in sera taken from patients with pre transplant diagnosis of PBC or PSC sera and controls against any of the bile duct tissues used.

**5.3.2.4 IgG and Hepatic Artery Analysis**

**IgG Antibodies and Chronic Rejection**

Two antigens were identified by antisera that discriminated between patients that developed chronic rejection (n=7) and controls (n=13; all others).

A 160kD antigen was found in a greater proportion of post-transplant sera from chronic rejectors compared to controls for all hepatic artery tissues tested. This difference was pronounced for 'chronically rejected' hepatic artery (see figure 5.8). It is possible that this antibody conferred a risk for chronic rejection and that a 160 kD antigen was upregulated in hepatic artery by chronic rejection.

**Figure 5.8**  Proportion of Sera from Patients that Developed Chronic Rejection (CR) and controls (NON-CR) that Contained IgG Antibodies to a 160 kD Antigen in 'Chronically Rejected Hepatic Artery'
An 85kD antigen was discriminatory in all hepatic artery tissue; discrimination was not enhanced with 'chronically rejected' hepatic artery but this tissue is shown in figure 5.9.

Figure 5.9  Proportion of Sera from Patients that Developed Chronic Rejection (CR) and controls (NON-CR) that Contained IgG Antibodies to an 85 kD Antigen in 'Chronically Rejected Hepatic Artery'

<table>
<thead>
<tr>
<th>Percent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

85 kD PRE 85 kD POST

IgG Antibodies and Active CMV Infection
No notable differences were seen between antibodies detected in sera taken from patients that developed active CMV infection and controls.

IgG Antibodies and PBC and PSC
No notable differences were seen between the antibodies detected in sera taken from patients with pre transplant diagnosis of PBC or PSC sera and controls against any of the hepatic artery tissues used.
5.3.2.5 Summary for Phase I

Overall

- Sera from liver transplant patients contained antibodies to 18 bile duct antigens and 16 hepatic artery antigens.

- Sera from two normal subjects contained antibodies to four bile duct and four hepatic artery antigens suggesting that the majority of antibodies identified in liver transplant recipients were associated with liver disease.

IgA Reactivity with Bile Duct

- Antibody to two antigens (94 kD and 39 kD) discriminated between chronic rejectors and others; this discrimination was more pronounced using 'chronically rejected, CMV infected' bile duct. Two normal sera did not contain antibodies to either antigen.

- Antibody to a 44 kD antigen discriminated between patients with active CMV infection and others; this was only observed using 'chronically rejected, CMV infected' bile duct. Unfortunately, CMV infected bile duct tissue which was not chronically rejected was not available.

- No notable differences were observed between sera from patients with pre transplant diagnosis of PBC or PSC and controls for any of the tissue screened.

IgG Reactivity with Hepatic Artery

- Antibody to two antigens (160 kD and 85 kD) discriminated between chronic rejectors and others; for the 160 kD antigen, this was most pronounced using 'chronically rejected' hepatic artery.

- No notable differences were observed between sera from patients with active CMV infection and others for any of the tissue screened.

- No notable differences were observed between sera from patients with pre transplant diagnosis of PBC or PSC and controls for any of the tissue screened.
5.3.3 Phase II

Further sera were screened against a limited panel of tissues which are listed below and results from both phases were considered in the statistical approach. Therefore, the results presented here were generated from screening sera from 38 patients that underwent 43 liver transplants (38 pretransplant sera and 43 posttransplant sera; see table 5.1).

These results were subject to four rounds of statistical analysis separating sera from patients that developed chronic rejection (CR-sera), that developed active CMV infection (CMV sera) and patients with pretransplant diagnosis of PBC (PBC sera) or PSC (PSC sera). Differences between the test group and control group were analysed using Fishers exact test. Caution was used when interpreting these results because they were generated from a relatively small number of patients (type II error) and multiple testing was performed.

5.3.3.1 IgA Antibodies to Bile Duct Tissue

Tissue Panel

The following tissue was tested:

1. Bile duct from an explanted liver that was lost to chronic rejection and was infected by CMV ('chronically rejected, CMV infected' bile duct; see phase I)

2. Bile duct taken from a 'cut down' donor liver ('normal' bile duct)

IgA Antibodies and Chronic Rejection

Comparison of patients that developed chronic rejection and controls for antibodies reactive with antigens from Western blotted chronically
rejected, CMV infected bile duct (CR/CMV bile duct) and normal bile duct showed that antibodies antigens of 94kD and 39kD were discriminatory (see tables 5.2, 5.3, 5.4 and 5.5).

IgA Antibodies to a 94kD Bile Duct Antigen

Table 5.2 Distribution of Sera Containing IgA Antibodies to a 94kD Antigen Present in Chronically Rejected, CMV Infected Bile Duct (CR/CMV bile duct) or Normal Bile Duct

<table>
<thead>
<tr>
<th>Size kD</th>
<th>CR/CMV bile duct Pretransplant sera</th>
<th>Post transplant sera</th>
<th>Normal bile duct Pretransplant sera</th>
<th>Post transplant sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>CR sera (n=10)</td>
<td>Others (n=28)</td>
<td>CR sera (n=10)</td>
<td>Others (n=33)</td>
</tr>
<tr>
<td></td>
<td>50% (p&lt;0.03)</td>
<td>11% (p=0.42)</td>
<td>0%</td>
<td>39% (p&lt;0.02)</td>
</tr>
<tr>
<td></td>
<td>10% (p=0.42)</td>
<td>12% (p=0.42)</td>
<td>10%</td>
<td>21% (p=0.29)</td>
</tr>
</tbody>
</table>

p values were calculated from the difference between CR sera and other sera using Fishers exact test.

Antibodies to a 94 kD antigen in 'chronically rejected, CMV infected' bile duct were also found significantly more often in pre-transplant sera from patients that developed chronic rejection (CR sera) compared to control (non-CR) sera when screening 'chronically rejected, CMV infected' bile duct (CR/CMV). These antibodies were not detected in any chronic rejectors after transplantation. Interestingly, prior chronic rejection was not a risk factor for chronic rejection after retransplantation in this group of patients (see Chapter 4).

However, autoantibodies to a 94 kD antigen in normal bile duct were found in pre-transplant non-CR sera but not in CR sera.
This result suggests that either the process of chronic rejection or CMV infection leads to upregulation of a 94kD antigen. These two effects may be distinguished by analysing closely the results generated in phases I and II when sera from six patients that developed chronic rejection were screened against a panel of bile duct tissues (see table 5.3).

Table 5.3 Presence (+) or Absence (-) of IgA Antibodies to a 94kD Antigen Present in Various Bile Duct Tissues (CR/CMV, CR, Tx or Normal) for Sera from Six Patients that Developed Chronic Rejection

<table>
<thead>
<tr>
<th>Patient Number (See Appendix 1)</th>
<th>Pre transplant Sera</th>
<th>Post transplant Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR/CMV</td>
<td>CR</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.3 shows results from screening four bile duct tissues which are defined as follows: CR/CMV= 'chronically rejected/CMV infected' bile duct, CR= 'chronically rejected' bile duct without CMV infection, Tx= 'transplanted' bile duct, Normal= 'normal' bile duct. Full descriptions of these tissues are given above (sections 5.2.3 and 5.2.4). The results of screening both pre and post transplant sera against these tissues are shown (+ indicates presence and - indicates absence of antigen identification by antisera) for six chronic rejectors (patient numbers 1, 2, 3, 5, 6 and 13) who are described in Appendix 1.

For pre transplant sera, 'chronically rejected' bile duct without CMV infection had a similar immunogenicity with respect to a 94kD antigen when compared to 'chronically rejected/CMV infected' bile duct; both bile
ducts were more immunogenic with respect to a 94kD antigen compared to 'transplanted' bile duct or 'normal' bile duct. This result suggested that the process of chronic rejection upregulated expression of a 94kD antigen in bile duct tissue and that this upregulation was not mediated or enhanced by CMV infection.

IgA Antibodies to a 39kD Bile Duct Antigen

Table 5.4 Distribution of Sera Containing IgA Antibodies to a 39 kD Antigen Present in 'Chronically Rejected, CMV Infected' Bile Duct (CR/CMV bile duct) or 'Normal' Bile Duct

<table>
<thead>
<tr>
<th>Size kD</th>
<th>CR/ CMV bile duct</th>
<th>Normal bile duct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretransplant sera</td>
<td>Post transplant sera</td>
</tr>
<tr>
<td>CR sera (n=10)</td>
<td>Others (n=28)</td>
<td>CR sera (n=10)</td>
</tr>
<tr>
<td>39</td>
<td>80% (p=0.01)</td>
<td>90% (p=0.002)</td>
</tr>
</tbody>
</table>

p values were calculated from the difference between CR sera and other sera using Fishers exact test.

Antibodies to the 39 kD antigen were found in a significantly larger proportion of pre transplant and post transplant CR sera compared to non-CR sera. Antibodies to a 39 kD antigen in 'chronically rejected, CMV infected' bile duct were highly prevalent among patients that developed chronic rejection (ie. harboured post transplant by 90% of chronic rejectors) and their detection may be potentially useful for diagnosis of chronic rejection.

A similar trend was seen for pre transplant CR sera against normal bile duct but significance was not reached.
This result suggested that either the process of chronic rejection or CMV infection led to upregulation of a 39 kD antigen. These two effects may be distinguished by analysing closely the results generated in phases I and II when sera from six patients that developed chronic rejection were screened against a panel of bile duct tissues (see table 5.5).

Table 5.5  Presence (+) or Absence (-) of IgA Antibodies to a 39 kD Antigen Present in Various Bile Duct Tissues (CR/CMV, CR, Tx or Normal) for Sera from Six Patients that Developed Chronic Rejection

<table>
<thead>
<tr>
<th>Patient Number (see Appendix 1)</th>
<th>Pre transplant Sera</th>
<th>Post transplant Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR/CMV</td>
<td>CR</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 5.5 shows results from screening four bile duct tissues which are defined as follows: CR/CMV= 'chronically rejected/ CMV infected' bile duct, CR= 'chronically rejected' bile duct without CMV infection, Tx= 'transplanted' bile duct, Normal= 'normal' bile duct. Full descriptions of these tissues are given above (sections 5.2.3 and 5.2.4). The results of screening both pre and post transplant sera against these tissues are shown (+ indicates presence and - indicates absence of antigen recognised by antisera) for six chronic rejectors (patient numbers 1, 2, 3, 5, 6 and 13) who are described in Appendix 1.

For both pre and post transplant sera, 'chronically rejected' bile duct had similar immunogenicity with respect to a 39 kD antigen when compared to 'chronically rejected/ CMV infected' bile duct. However, both bile ducts were more immunogenic with respect to a 39 kD antigen compared to
'transplanted' bile duct or 'normal' bile duct. This result suggests that the process of chronic rejection upregulated expression of a 39 kD antigen in bile duct tissue and that this upregulation was not mediated or enhanced by CMV infection.

**IgA Antibodies to a 94kD or 39kD Bile Duct Antigen**

When the results shown in tables 5.2 and 5.4 were combined for analysis of 'chronically rejected/ CMV infected' bile duct it was shown that all patients that developed chronic rejection contained antibodies to a 94kD antigen or to a 39kD antigen either pre or post transplant.

**IgA Antibodies and Active CMV Infection**

The presence of IgA antibodies was compared between patients with active CMV infection and controls after Western blotting 'chronically rejected, CMV infected' bile duct and 'normal' bile duct (see table 5.6).

**Table 5.6 Distribution of Sera Containing IgA Antibodies to a 44 kD Antigen Present in Chronically Rejected, CMV Infected Bile Duct (CR/CMV bile duct) or Normal Bile Duct**

<table>
<thead>
<tr>
<th>Size kD</th>
<th>CR/ CMV bile duct</th>
<th>Normal bile duct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretransplant sera</td>
<td>Post transplant sera</td>
</tr>
<tr>
<td>44</td>
<td>CMV (n=27) 11% (p=0.435)</td>
<td>Others (n=11) 10%</td>
</tr>
<tr>
<td></td>
<td>CMV (n=27) 4% (p=0.71)</td>
<td>Others (n=11) 0%</td>
</tr>
</tbody>
</table>

A 44 kD antigen was identified more often with post transplant sera from patients with active CMV infection compared to controls when sera were screened against 'chronically rejected, CMV infected' bile duct but this
result was not significant. Only one serum sample contained antibodies that recognised a 44 kD antigen in 'normal' bile duct.

Furthermore, results generated in phase I of this study (see section 5.3.2.4 and figure 5.7) showed that, for a proportion of the patients used for phase II statistical analysis, antibodies to a 44 kD antigen were discriminatory post transplantation when sera was screened against 'chronically rejected, CMV infected' bile duct but that this trend was not evident when sera was screened against 'chronically rejected' (uninfected) bile duct or 'transplanted' bile duct (explanted, grafted liver that was not chronically rejected). This suggested that CMV infection of bile duct tissue was responsible for upregulation of a 44 kD antigen.

It is possible that the 44kD antigen was a CMV protein but this must be reconciled with the fact that phase I analysis showed that antibodies to a 44 kD antigen in CMV-infected fibroblasts did not discriminate between patients that developed active CMV infection and controls. Antigenic differences between the laboratory strain (AD169) used to infect fibroblasts in vitro and the clinical strain which infected bile duct tissue in vivo may account for this result.

Another possibility was that the 44kD antigen was a cellular protein that was upregulated by CMV infection and was specific to bile duct tissue.

However, identification of a 44kD antigen with antisera was not associated with the presence of chronic rejection (see above) and was therefore not investigated further.
**IgA Antibodies and Pre-transplant Diagnosis of PSC**

The presence of antibodies was compared between patients with pre transplant diagnosis of PSC ('PSC patients') and controls after Western blotting chronically rejected, CMV infected bile duct or normal bile duct. Here, controls did not include those patients with a pre transplant diagnosis of PBC.

Antibodies directed to antigens of 145, 134 and 62 kD were most commonly (>40% sera) found in pre transplant sera from 'PSC patients'. However, only the 134 kD antigen was discriminatory (see table 5.7).

### Table 5.7 Distribution of Sera Containing IgA Antibodies to a 134 kD Antigen Present in 'Chronically Rejected, CMV Infected' Bile Duct (CR/CMV bile duct) or 'Normal' Bile Duct

<table>
<thead>
<tr>
<th>Size kD</th>
<th>CR/ CMV bile duct</th>
<th>Normal bile duct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretransplant sera</td>
<td>Post transplant sera</td>
</tr>
<tr>
<td></td>
<td>PSC (n=6)</td>
<td>Others (n=32)</td>
</tr>
<tr>
<td>134</td>
<td>100% (p&lt;0.05)</td>
<td>53% (p&lt;0.05)</td>
</tr>
</tbody>
</table>

A 134 kD antigen was identified by a significantly higher proportion of both pre and post transplant from 'PSC patients' when screened against 'chronically rejected, CMV infected' bile duct; before transplantation, all 'PSC patients' harboured antibody to the 134 kD antigen. However, this was not observed when sera was screened against 'normal' bile duct.

Useful analysis of a 134 kD antigen was not possible in phase I where only one PSC patient (number 31) was studied. However, pre and post
transplant sera from this PSC patient was shown to contain antibodies to a 134kD antigen of 'PSC bileduct' (ie. bile duct from a liver explanted after PSC).

Antibodies to a 134 kD antigen were not associated with chronic rejection (see above).

**IgA Antibodies and Pretransplant Diagnosis of PBC**

The presence of antibodies was compared between patients with pre-transplant diagnosis of PBC ('PBC patients') and controls after Western blotting 'chronically rejected, CMV infected' bile duct or 'normal' bile duct. Here, controls did not include those patients with a pre transplant diagnosis of PSC.

Antibodies directed to antigens of 145, 134, 99 and 44 kD were most commonly (>40% sera) found in the pre transplant sera of 'PBC patients'. However, only the 44 kD antigen was discriminatory (see table 5.8).

**Table 5.8 Distribution of Sera Containing IgA Antibodies to a 44 kD Antigen Present in 'Chronically Rejected, CMV Infected' Bile Duct (CR/CMV bile duct) or 'Normal' Bile Duct**

<table>
<thead>
<tr>
<th></th>
<th>CR/CMV bile duct</th>
<th>Normal bile duct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretransplant sera</td>
<td>Post transplant sera</td>
</tr>
<tr>
<td>Size kD</td>
<td>PBC (n=9)</td>
<td>Others (n=29)</td>
</tr>
<tr>
<td>44</td>
<td>56%  (p=0.009)</td>
<td>10%</td>
</tr>
</tbody>
</table>
Antibodies to a 44 kD antigen in 'chronically rejected, CMV infected' bile duct were detected in a significantly higher proportion of pre transplant sera from 'PBC patients' compared to controls. However, this was not observed when sera were screened against normal bile duct. Discrimination by identification of a 44 kD antigen with antisera was not apparent in phase I.

However, antibody to a 44kD antigen was not associated with the presence of chronic rejection (see above).

**Summary : IgA Antibodies Reactive to Bile Duct**

**Using Chronically Rejected, CMV Infected Bile Duct**

**IgA Antibodies to Bile Duct Antigens and Chronic Rejection**
- Antibody to a 94 kD antigen discriminated between pretransplant sera from chronic rejectors and 'others'; antibodies to this antigen were lost by all chronic rejectors after transplantation. Results from phase I suggested that a 94 kD bile duct antigen was upregulated by chronic rejection but not by CMV infection of bile duct.

- Antibody to a 39 kD antigen discriminated between sera from chronic rejectors and 'others'; this was observed before and after transplantation. Antibodies to a 39 kD antigen of 'chronically rejected, CMV infected' bile duct were prevalent among patients that developed chronic rejection and may be useful for diagnosis of chronic rejection. Results from phase I suggested that a 39 kD bile duct antigen was upregulated by chronic rejection but not by CMV infection of bile duct.

**IgA Antibodies to Bile Duct Antigens and Active CMV Infection**
- The appearance of antibodies to a 44 kD antigen after transplantation discriminated between patients that had developed active CMV infection and others.

**IgA Antibodies to Bile Duct Antigens and Pretransplant Diagnosis of PBC**
- Antibody to a 44 kD antigen also discriminated between pretransplant sera of PBC patients and others.
IgA Antibodies to Bile Duct Antigens and Pretransplant Diagnosis of PSC

• Antibody to a 134 kD antigen discriminated between sera from PSC patients and others both before and after transplantation; all PSC patients harboured antibody to this antigen before transplantation.

Using Normal Bile Duct

• Screening sera against normal bile duct was never significantly discriminatory for chronic rejection, active CMV infection or pre transplant diagnosis of PSC or PBC; normal bile duct was less immunogenic than chronically rejected bile duct.

5.3.3.2 IgG Antibodies to Hepatic Artery Tissue

Tissue Panel

The following tissue was screened:

1. Hepatic artery from an explanted liver that was lost to chronic rejection but was not infected by CMV ('chronically rejected' hepatic artery; see phase I)

2. Hepatic artery taken from a 'cut down' donor liver ('normal' hepatic artery)

IgG Antibodies and Chronic Rejection

The presence of antibodies was compared between chronic rejectors and controls after Western blotting 'chronically rejected' and 'normal' hepatic artery. Identification of 160 kD and 85 kD antigens by antisera was discriminatory (see tables 5.9, 5.10, 5.11 and 5.12).
IgG Antibodies to a 160kD Antigen

Table 5.9 Distribution of Sera Containing IgG Antibodies to a 160 kD Antigen Present in 'Chronically Rejected' Hepatic Artery (CR Hepatic Artery) or 'Normal' Hepatic Artery

<table>
<thead>
<tr>
<th>Size kD</th>
<th>CR Hepatic Artery</th>
<th>Normal Hepatic Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretransplant sera</td>
<td>Post transplant sera</td>
</tr>
<tr>
<td>CR sera (n=10)</td>
<td>Others (n=28)</td>
<td>CR sera (n=10)</td>
</tr>
<tr>
<td>160</td>
<td>50% (p=0.08)</td>
<td>21% (p=0.07)</td>
</tr>
</tbody>
</table>

Antibodies to a 160 kD antigen in 'chronically rejected' hepatic artery were found more often in pre transplant and post transplant sera from patients that developed chronic rejection (CR sera) compared to others. However, these differences were not significant.

Screening against 'normal' hepatic artery showed that pre transplant antibodies to a 160 kD antigen were present in a higher proportion of patients that developed chronic rejection compared to others (this difference was not significant) but were lost from all chronic rejectors after transplantation.

The identification of a 160 kD antigen by antisera after Western blot screening of various hepatic artery tissues was analysed to determine whether the process of chronic rejection altered the immunogenicity of hepatic artery for a 160 kD antigen; results are shown for the six patients
that developed chronic rejection that were studied in phase I (see table 5.10).

Table 5.10  Presence (+) or Absence (-) of IgG Antibodies to a 160kD Antigen Present in Various Hepatic Artery Tissues (CR, Tx or Normal) for Sera from Six Patients that Developed Chronic Rejection

<table>
<thead>
<tr>
<th>Patient Number (see Appendix 1)</th>
<th>Pre transplant Sera</th>
<th>Post transplant Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR</td>
<td>Tx</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.10 shows results from screening four hepatic artery tissues which are defined as follows: CR= 'chronically rejected' hepatic artery, Tx= 'transplanted' hepatic artery, Normal= 'normal' hepatic artery. Full descriptions of these tissues are given above (sections 5.2.3 and 5.2.4). The results of screening both pre and post transplant sera against these tissues are shown (+ indicates presence and - indicates absence of antigen recognised by antisera) for six chronic rejectors (patient numbers 1, 2, 3, 5, 6 and 13) who are described in Appendix 1.

The results shown in table 5.10 for pre transplant sera suggested that the process of chronic rejection did not upregulate a 160 kD antigen. In contrast, post transplant sera identified a 160 kD antigen only in 'chronically rejected' hepatic artery. These results may be consolidated if the 160 kD antigens identified in these tissues were not identical; here, post transplant upregulation of antibodies to a 160 kD antigen in 'chronically rejected' hepatic artery may occur simultaneously with downregulation of
antibodies to a 160 kD antigen present in 'transplanted' and/or 'normal' hepatic artery.

**IgG Antibodies to an 85 kD Antigen**

Table 5.11 Distribution of Sera Containing IgG Antibodies to an 85 kD Antigen Present in 'Chronically Rejected' Hepatic Artery (CR Hepatic Artery) or 'Normal' Hepatic Artery

<table>
<thead>
<tr>
<th></th>
<th>CR Hepatic Artery</th>
<th>Normal Hepatic Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretransplant sera</td>
<td>Post transplant sera</td>
</tr>
<tr>
<td>Size kD</td>
<td>CR sera (n=10)</td>
<td>Others (n=28)</td>
</tr>
<tr>
<td>85</td>
<td>50%</td>
<td>11% (p&lt;0.04)</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>25% (p=0.11)</td>
</tr>
</tbody>
</table>

Antibodies to the 85 kD antigen were found in a larger proportion of pre transplant and post transplant sera from patients that developed chronic rejection (CR sera) compared to controls (non-CR sera). A similar trend was seen after screening sera against normal bile duct but significance was not reached.

The identification of an 85 kD antigen by antisera after Western blot screening of various hepatic artery tissues was analysed to determine whether the process of chronic rejection altered the immunogenicity of hepatic artery for an 85 kD antigen; results are shown for the six patients that developed chronic rejection that were studied in phase I (see table 5.12).
Table 5.12  Presence (+) or Absence (-) of IgG Antibodies to an 85 kD Antigen Present in Various Hepatic Artery Tissues (CR, Tx or Normal) for Sera from Six Patients that Developed Chronic Rejection

<table>
<thead>
<tr>
<th>Patient Number (see Appendix 1)</th>
<th>Pre transplant Sera</th>
<th>Post transplant Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR</td>
<td>Tx</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.12 shows results from screening three hepatic artery tissues which are defined as follows: CR= 'chronically rejected' hepatic artery, Tx= 'transplanted' hepatic artery, Normal= 'normal' hepatic artery. Full descriptions of these tissues are given above (sections 5.2.3 and 5.2.4). The results of screening both pre and post transplant sera against these tissues are shown (+ indicates presence and - indicates absence of antigen recognised by antisera) for six chronic rejectors (patient numbers 1, 2, 3, 5, 6 and 13) who are described in Appendix 1.

The results shown in table 5.12 suggested that the process of chronic rejection did not upregulate an 85 kD antigen; only one patient (patient 5) identified an 85kD antigen which was exclusive to 'chronically rejected' hepatic artery.

IgG Antibodies to a 160kD or 94kD Hepatic Artery Antigen

Comparison of tables 5.10 and 5.12 shows that, for patients that developed chronic rejection, the presence of antibodies to a 160kD antigen was usually associated with antibodies to an 85kD antigen. All patients (1, 2, 5
that harboured antibodies to a 160kD antigen in 'chronically rejected' hepatic artery also harboured antibodies to an 85kD antigen in this tissue.

Furthermore, only one patient that developed chronic rejection (patient 13) did not harbour IgG antibodies to either a 160 kD or an 85 kD hepatic artery antigen.

**IgG Antibodies and Active CMV Infection**
The presence of antibodies was compared between sera from patients with active CMV infection and controls after Western blotting 'chronically rejected' and 'normal' hepatic artery. None of the antigens identified were discriminatory.

**IgG Antibodies and PSC**
The presence of antibodies was compared between sera from patients with pre transplant diagnosis of PSC (PSC sera) and controls after Western blotting 'chronically rejected' and 'normal' hepatic artery. Here, controls did not include those patients with a pre transplant diagnosis of PBC.

For both tissues screened, antibodies in PSC sera were most commonly directed to antigens of 210, 159, 109, 78 and 75 kD; identification of these antigens with antisera was not discriminatory.

**IgG Antibodies and PBC**
The presence of autoantibodies was compared between sera from patients with pre-transplant diagnosis of PBC (PBC sera) and controls after Western blotting 'chronically rejected' and 'normal' hepatic artery. Here, controls did not include those patients with a pretransplant diagnosis of PSC.
For chronically rejected and normal hepatic artery, antibodies in PBC sera were most commonly directed to antigens of 210, 185, 140, 109, 78 and 75 kD; identification of these antigens with antisera was not discriminatory.

Summary: IgG Antibodies to Hepatic Artery

IgG Antibodies to Hepatic Artery Antigens and Chronic Rejection

- Screening against 'chronically rejected' hepatic artery showed that a greater proportion of chronic rejectors harboured antibodies that identified a 160 kD antigen compared to controls; this was observed before and after transplantation. This trend did not reach significance.

- Similarly, screening against normal hepatic artery showed that identification of a 160 kD antigen was discriminatory pretransplant. However, antibody to this 160 kD antigen was not detected in chronic rejectors after transplantation.

- Identification of a 85 kD antigen discriminated between sera from chronic rejectors and 'others' both pre- and post-transplant; this trend was observed after screening against chronically rejected and normal hepatic artery.

- Analysis of results from phase I suggested that the presence of antibodies to a 160kD antigen was associated with the presence of antibodies to an 85 kD antigen in 'chronically rejected' hepatic artery.

IgG Antibodies to Hepatic Artery Antigens and Active CMV Infection

- IgG antibodies to chronically rejected or normal hepatic artery did not discriminate between sera from patients with active CMV and 'others'.

IgG Antibodies to Hepatic Artery Antigens and Pre transplant Diagnosis of PBC

- IgG antibodies to chronically rejected or normal hepatic artery did not discriminate between sera from 'PBC patients' and 'others'.

IgG Antibodies to Hepatic Artery Antigens and Pre transplant Diagnosis of PSC

- IgG antibodies to chronically rejected or normal hepatic artery did not discriminate between sera from 'PSC patients' and 'others'
5.3.4 Lack of Cross-Reactivity Between Cellular Antigens and CMV-Specific Antibodies

Chronically rejected hepatic artery was blotted and polyclonal antibodies to CMV (raised in goat) were applied before application of sera containing antibodies of interest. This enabled blocking of endogenous antibody binding and binding of CMV specific antibodies to cellular proteins to be observed (see figure 5.10).

The banding pattern obtained after incubation of blots with sera, which had previously been shown to contain autoantibodies to the 85 kD and 160 kD hepatic artery antigens, was unaltered by prior incubation of the blots with polyclonal antibodies directed to CMV or adenovirus i.e. polyclonal antibodies to CMV did not block the binding of antibodies of interest to this tissue.

However, detection of goat IgG showed that interaction did take place between CMV polyclonal antibodies and 9 hepatic artery proteins of 253, 245, 210, 185, 177, 167, 151, 145 and 78 kD molecular weight; 5 of which (253, 245, 210, 185 and 78 kD) were previously shown to be targets for antibodies in vivo (see section 5.3.2). Interaction also took place between adenovirus polyclonal antibodies and autoantigens of 78 and 85 kD. However, these interactions were non-specific and occurred, with low avidity, only when antibodies were added at high titre (1:100); this interaction did not prevent binding of human antibodies. An identical experiment was performed to investigate possible cross-reactivity between antibodies to CMV and the bile duct antigens of interest (94 and 39 kD; results not shown). No such cross-reactivity was seen.
Figure 5.10

Autoantigens Present in Hepatic Artery do Not Cross-react with Antibodies Raised to CMV Proteins

Blocking Antibody:  
Secondary Antibody: α-human IgG  
→ Adenovirus (1:1000)  
→ Adenovirus (1:500)  
→ Adenovirus (1:100)  
→ CMV (1:1000)  
→ CMV (1:500)  
→ CMV (1:100)  

Blocking Antibody:  
Secondary Antibody: α-goat IgG  
→ Adenovirus (1:1000)  
→ Adenovirus (1:500)  
→ Adenovirus (1:100)  
→ CMV (1:1000)  
→ CMV (1:500)  

Figure 5.10 shows the result of a 'blocking experiment' designed to investigate whether IgG autoantigens of interest cross-react with antibodies raised against CMV. Chronically rejected hepatic artery was bled while the blots were blocked; blocking solution contained various concentrations of polyvalent antibodies to adenovirus or CMV (raised in goat) as indicated. Sera was now applied; this sera was known to contain autoantibodies to the 160 KD and 85 KD hepatic artery antigens which are the bands of interest. Subsequent development was carried out after application of a secondary antibody; this comprised anti (α)-human IgG or anti (α)-goat IgG. The latter was used to directly identify cellular proteins that interact with antibodies to CMV antigens whereas the former was used to detect 'blocking' of cellular antigenic sites by antibodies raised to CMV antigens.
Summary

- Hepatic artery antigens of interest (160 and 85 kD) did not cross react with antibodies raised against cytomegalovirus (AD169) proteins in goats.

- Bile duct antigens of interest (94 and 39 kD) did not cross react with antibodies raised against cytomegalovirus (AD169) proteins in goats.
5.4 DISCUSSION

The results presented in this chapter underwent 4 rounds of univariate analysis and each antigen that was identified with antisera was analysed separately. It is possible that some of the significant trends observed were due to random events. Therefore, the findings of this chapter must be interpreted with caution. However, these findings set up interesting hypotheses which should be tested in a larger study.

5.4.1 Conclusions

• The hypothesis that antibodies to bile duct or hepatic artery initiate or enhance chronic rejection was upheld. Two antibodies to chronically rejected, CMV infected bile duct (94 and 39 kD) and 2 antibodies to chronically rejected hepatic artery (160 and 85 kD) discriminated between chronic rejectors and 'others'. It is of interest that these antibodies were often detected prior to transplant.

• Conflicting evidence was generated for the hypothesis that active cytomegalovirus (CMV) infection of hepatic artery or bile duct upregulates the expression of target antigens. Chronically rejected, CMV infected bile duct was more immunogenic than its chronically rejected (uninfected) (phase I) and normal (phase II) counterparts. However, CMV infected fibroblasts were not more immunogenic than uninfected fibroblasts (phase I).

• The hypothesis that active CMV infection upregulates the production of antibodies involved in chronic rejection was not upheld. IgA antibody to a 44kD bile duct antigen identified patients that developed active CMV infection but was not associated with chronic rejection.

• The hypothesis that antibodies produced to CMV are cross reactive with cellular antigens involved in chronic rejection was not upheld.

• The hypothesis that autoantibodies associated with primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) are present after transplantation for these diseases and are associated with chronic rejection was not upheld. Antibodies to bile duct (44 and 134 kD) were shown to discriminate PBC sera and PSC sera from 'other sera' respectively, but these antibodies were not associated with chronic rejection.
5.4.2 Humoral Immunity and Chronic Rejection

Four antibodies were found in a significantly higher proportion of chronic rejectors compared to controls. As far as I am aware, similar work has not been previously reported in the context of liver transplantation.

5.4.2.1 Two Antibodies to Bile Duct were Associated with Chronic Rejection

IgA Antibodies to a 39 kD Bile Duct Antigen

Pre and post transplant sera from chronic rejectors were associated with IgA antibody to a 39 kD protein; this result was only significant using 'chronically rejected, CMV infected' bile duct. A similar trend (but without significance) was seen after screening normal bile duct but less chronic rejectors were shown to harbour antibody.

It was therefore proposed that the reactivity of a 39 kD protein was enhanced by CMV infection or by the process of chronic rejection.

Indeed, a number of cellular proteins (54, 55, 366, 214, 264, 265, 266, 267, 268; see sections 1.3.3.1, 1.9.2 and 1.9.3) and cellular antigens (312, 313, 314; see section 1.9.5) have been shown to be upregulated after CMV infection.

However, the hypothesis that CMV infection led to upregulation of a 39 kD bile duct antigen was not supported by results generated in phase I. Here, 'chronically rejected' bile duct and 'chronically rejected, CMV infected' bile duct were similarly immunogenic with respect to the 39kD
antigen when immunoblotted using sera from six patients that developed chronic rejection.

Another possibility considered was that chronic rejection may have enhanced the reactivity of a 39 kD bile duct antigen. Indeed, chronic rejection of liver grafts is associated with the increased surface expression of many proteins which may, or may not, be immunogenic; these include HLA molecules (213, 287), adhesion molecules (374) and lymphokines (373).

The hypothesis that chronic rejection was associated with upregulation of a 39 kD bile duct antigen was supported by results generated in phase I of this study; both 'chronically rejected' bile duct and 'chronically rejected, CMV infected' bile duct were more immunogenic than 'transplanted' or 'normal' bile duct with respect to a 39 kD antigen using sera from six patients that developed chronic rejection.

A 39 kD antigen identified in 'chronically rejected, CMV infected' bile duct was not a CMV protein; identification of this antigen was not exclusive to CMV infected tissue. However, it was proposed that antibodies to the 39 kD antigen were cross-reacting with CMV proteins but this possibility was excluded in a later experiment (see section 5.3.4).

**IgA Antibodies to a 94 kD Bile Duct Antigen**

Similarly, pretransplant sera from chronic rejectors was associated with IgA antibodies to a 94 kD antigen in 'chronically rejected, CMV infected' bile duct. Reactivity was enhanced in this tissue compared to 'normal' tissue and it was proposed that the process of chronic rejection or CMV infection was associated with upregulation of a 94 kD bile duct antigen.
This was not supported by results generated in phase I; 'chronically rejected' bile duct and 'chronically rejected, CMV infected' bile duct were similarly immunogenic with respect to a 94 kD antigen using sera from six patients that developed chronic rejection.

Phase I results suggested that chronic rejection led to upregulation of a 94 kD bile duct antigen; both 'chronically rejected' bile duct and 'chronically rejected, CMV infected' bile duct were more immunogenic than 'transplanted' or 'normal' bile duct with respect to a 94 kD antigen using sera from six patients that developed chronic rejection.

Intriguingly, patients that developed chronic rejection became seronegative for antibodies to a 94 kD antigen in 'chronically rejected, CMV infected' bile duct, after transplantation. One possibility is that immunosuppression following transplantation led to downregulation of production of this antibody.

However, it is possible that this result was due to deposition of this antibody in the grafted liver after transplantation. This does not account for the results observed after blotting normal bile duct with post transplant sera from patients who did not develop chronic rejection and in whom antibody was detected. However, it is possible that the 94 kD antigens detected in these two bile duct tissues are distinct.

5.4.2.2 Two Antibodies to Hepatic Artery were Associated with Chronic Rejection

Two IgG antibodies to hepatic artery were also associated with chronic rejection.
**IgG Antibodies to an 85 kD Hepatic Artery Antigen**

Pre transplant IgG antibodies to an 85 kD antigen in 'chronically rejected' hepatic artery were significantly associated with rejection; a similar trend was apparent post transplant but without significance. Similarly, IgG antibodies to an 85 kD antigen in 'normal' hepatic artery were associated with rejection (significance shown post transplant only).

Comparison of results from phases I and II using 'chronically rejected', 'transplanted' or 'normal' hepatic artery against sera from six patients that developed chronic rejection suggested that an 85 kD antigen was not upregulated by transplantation or by the process of chronic rejection.

Furthermore, phase I results showed that an 85 kD antigen was also present at high levels in fibroblasts but it is not known whether the 85 kD antigen detected in fibroblasts was identical to that identified in hepatic artery. If the 85 kD antigens in bile duct and fibroblasts were identical, this would not be necessarily inconsistent with an 'organ-specific' rejection process; it is possible that the **surface expression** of this protein was enhanced in hepatic artery during the rejection process. Indeed, Latif et al (1995) (309) found antibodies to 'housekeeping' proteins (heat shock protein-60 and -70) that were associated with heart graft rejection (see section 1.8.3.3.3).

**IgG Antibodies to a 160 kD Hepatic Artery Antigen**

Posttransplant IgG antibody to a 160 kD antigen in 'chronically rejected' hepatic artery was associated with rejection (this result did not reach significance). However, post-transplant antibodies from patients that developed chronic rejection did not bind 160 kD antigens present in 'transplanted' or 'normal' hepatic artery whereas antibodies from control...
patients were shown to bind a 160 kD antigen from these tissues. However, it is possible that the 160 kD antigens that were present in these tissues were antigenically distinct.

Results from phase I showed that IgG antibodies to a 160 kD hepatic artery antigen were usually associated with IgG antibodies to an 85 kD hepatic artery antigen.

5.4.2.3 Causality

A causal relationship between these antibodies and chronic rejection has not been established. The association between chronic rejection and antibodies after transplantation, as shown for IgG antibody to the 160 kD hepatic artery antigen, could be due to increased release of antigen after rejection mediated damage. However, the link between pre transplant antibodies, as shown for IgG antibody to the 85 kD hepatic artery antigen and IgA antibodies to 39 kD and 94 kD bile duct antigens, is more compelling.

Further work should be directed at establishing the importance of these antibodies; purification of a particular antibody could be carried out by absorbing-out all other antibodies (this could be conducted by incubating sera with Western blotted proteins with the antigen of interest removed). The purified antibodies could then be used to perform immunohistochemistry on sections of normal and chronically rejected bile duct. Similar work for renal grafts has shown that antibodies localise at sites of rejection (305, 307; see section 1.8.3.3.3) including endothelial tissue (307; see also 308, 312) which is also a primary site of rejection for liver grafts (191, 193).
The antibodies identified in this study were not associated with active CMV infection (see sections 5.3.3.1 and 5.3.3.2) and were, therefore, not studied further.

5.4.3 Active CMV Infection, Humoral Immunity and Chronic Rejection

None of the antigens identified by antisera were confined exclusively to CMV infected cells or tissue nor were any detected exclusively with sera from patients with active CMV infection. Therefore it is unlikely that any of the Western blot bands represented CMV specific antibody bound to CMV protein; note that this does not exclude crossreactive antibody interactions, i.e. CMV specific antibody bound to human antigen or visa versa.

CMV induced autoantibodies have been reported previously in non-transplant settings (312, 313); in this study, IgA antibody to a 44 kD bile duct antigen identified patients that developed active CMV infection but was not associated with chronic rejection.

5.4.4 Antibodies and Pretransplant Diagnosis of PBC or PSC

PBC is a disease that involves small bile ducts whereas chronic rejection involves medium to large sized bile ducts; therefore, antibodies associated with PBC (320; see section 1.9.6) would not necessarily be expected to be associated with chronic rejection or detected when tested against large bile duct as performed in this study.

Nevertheless, pretransplant diagnoses of PBC or PSC have been reported to be a risk factor for chronic rejection (200, 201, 203, 205) and this link was shown to be significant for patients with PBC in this study (see Chapter 4).
Several of the commonest antigens detected by antibodies present in PBC (39, 53 and 75 kD) and PSC (210, 109 and 62 kD) sera, during this study, have previously been shown to be associated with PBC (320) or PSC (317, 318) disease. However, in this study none of the antigens identified with antisera were confined exclusively to PBC diseased liver tissue or PSC diseased bile duct (see Phase I) nor were any detected exclusively with sera from patients PSC or PBC.

This lack of discrimination may be due to 'masking' by other antigens of similar size (a single Western blot band does not necessarily correspond to a single antigen). In addition, antigens that discriminate between PBC and non-PBC sera may be 'burnt out' in PBC-liver and PSC-bile duct by previous immune activity or blocked by previous antigen binding in vivo. The latter phenomenon was addressed in Phase II of this study when normal tissue was screened.

A similar lack of discrimination was seen after screening of normal tissue. The system of detection used was highly sensitive (alkaline phosphatase) which may account for detection of antibodies with specificity to 'PBC antigens' and 'PSC antigens' in individuals without either liver disease. A similar result has been reported previously for PSC (318).

Furthermore, the Western blotting technique used was performed under denaturing conditions in order to efficiently separate proteins by size; such conditions destroy secondary and tertiary structure. Antibodies that react in vivo with epitopes with complex structure will therefore not be detected in vitro using this technique. It is probable that such antibodies exist in vivo in association with PBC or PSC and were not detected in this study.
However, Western blotting did identify IgA antibody to a 44 kD antigen in 'chronically rejected, CMV infected'; this was found in a significantly higher proportion of PBC patients pretransplant and was lost by all PBC patients post-transplant. Furthermore, this result was mirrored, albeit without significance, after blotting 'normal' bile duct tissue. The loss of antibody after transplantation may be due to removal of the antigen source (i.e. the diseased liver). This antigen may be the 'liver-pancreas' antigen previously reported to be 48-52 kD in size and associated with autoimmune hepatitis, PBC and PSC (321).

IgG antibody to the 140 kD hepatic artery antigen is also associated with PBC and also disappears after transplant. This was not seen when chronically rejected tissue was screened and it is possible that the antigen was 'burnt out' or blocked in this tissue.

In contrast, PBC was significantly associated with the presence of IgG to the 78 kD hepatic artery antigen both before and after transplantation. However, none of the antibodies associated with PBC were associated with the incidence of chronic rejection (see section 5.4.1). Antibody to an antigen of similar size (74 kD) was found to be associated with PBC by Van de Water et al (1989) (320).

PSC was significantly associated with IgA to the 134 kD bile duct antigen; this was seen for pre- and post-transplant sera but persistence of this antibody after transplantation was not associated with the incidence of chronic rejection (see section 5.4.1).
5.4.5 Antigens of Interest did Not Cross-React with Polyclonal Antibodies to CMV

Sera previously shown to contain antibodies of interest to bile duct or hepatic artery proteins was applied to tissue after incubation with polyclonal antibodies to CMV or adenovirus. The latter antibodies were shown to bind a number of antigens but did not prevent binding of human antibodies. This suggests that binding of CMV and adenovirus antibodies occurs through weak, non-specific interactions that are readily displaced by stronger, specific binding of antibodies.

Antigens did not cross react with CMV antibodies. It is possible that the cross-reactive antibodies suggested by a previous study (60) were specific to a particular HLA molecule which was not "inherited" by the donor of this tissue.

Human CMV does not replicate in goats and therefore the goat polyclonal antibodies used here will have specificities to structural proteins only. It was not possible to test crossreactivity of human antibodies to non-structural CMV proteins using the techniques presented in this Chapter.
CHAPTER 6

CYTOMEGALOVIRUS ACTIVELY INFECTS BILE DUCT EPITHELIAL CELLS AND VASCULAR ENDOTHELIAL CELLS OF LIVER GRAFTS LOST TO CHRONIC REJECTION
6.1 Introduction

Active cytomegalovirus (CMV) infection of graft tissue has been associated with chronic rejection in a number of reports (225, 284). The Pittsburgh group found that HLA DR matching facilitated CMV hepatitis which was a risk factor for chronic rejection (225; see section 1.8.2.2).

The Kings College group investigated serial liver biopsy specimens for active CMV infection by *in situ* hybridisation (ISH); *persistent* CMV infection of liver grafts constituted a risk for chronic rejection (284; see section 1.8.2.3). In this report CMV infection of hepatocytes was observed; in contrast, the primary sites of damage during chronic rejection, ie. large bile ducts and hepatic artery (see section 1.8.1.2), were not studied. The authors suggest that CMV *indirectly* promoted or initiated damage of these latter tissues. However, screening of biopsy specimens allowed only limited analysis of the primary sites of chronic rejection because only small bile ducts and arterioles were present in these specimens.

In contrast, this chapter reports the results of screening sections taken from explanted livers that have been lost to chronic rejection. Sections that contained large or medium sized bile duct and/or hepatic artery were selected for screening for active CMV infection by *in situ* hybridisation (ISH) and immunohistochemistry.

Four patients who lost grafts to hepatic artery thrombosis were also included in this study because the pathology of this disease is similar to the ischaemic injury observed during early stages of chronic rejection of some grafts (191, 193; see section 1.8.1.2).
Hubscher 1991 (191) defined two criteria for the diagnosis of chronic rejection (see section 1.8.1.2):

1. Vanishing bile duct syndrome (VBDS)

VBDS is a progressive disorder which cannot be treated and leads to graft loss.

2. Obliterative vasculopathy of small and medium sized arteries

Arteries are also involved; endothelial damage leads to infiltration of the intima by foamy macrophages and occlusion of the lumen.

An example of the histopathological changes that are characteristic of chronic rejection is shown in figure 6.1 and important points are described below.
Figure 6.1  Cross-sections of Normal and Chronically Rejected Portal Tracts

a  Normal portal tract

b  Chronically rejected portal tract

Figure 6.1 shows a cross-section of a normal portal tract (a) comprising bile duct (BD), hepatic artery (HA) and portal vein (PV). This is shown above a chronically rejected portal tract (b) which comprised ‘vanished’ bile duct (VBD), hepatic artery (HA) and portal vein (PV). Here, the ‘vanished’ bile duct can be identified by its position, adjacent to hepatic artery and portal vein, and shape; no bile duct epithelial cells remain. The hepatic artery is infiltrated by foamy macrophages (FM) which occlude the lumen.
Hypothesis

- Cytomegalovirus (CMV) may actively infect vascular endothelial and bile duct epithelial cells and may initiate or enhance chronic rejection at these sites.

6.2 Patients, Materials and Methods

6.2.1 Patients

Patients were selected initially according to the criteria that all provided more than one sample (serum, whole blood and urine) per week for more than two months after transplant (see section 2.2.6). Samples were tested in parallel by PCR, DEAFF and culture (see Chapter 2).

From this sub-group, liver graft sections from 29 liver transplant recipients were examined. Fifteen explanted grafts, from which sections were available, were selected for this study; eight and four patients lost their graft to chronic rejection (CR) or hepatic artery thrombosis (HAT) respectively.

The control group comprised three patients that lost grafts to other diseases (see Appendix 3) and 14 patients that retained their grafts. This highly selected group was biased towards patients with evidence of active CMV infection and comprised ten patients that developed active CMV infection as detected by serum or urine PCR and /or DEAFF testing (see Chapters 2 and 3) and four patients that remained negative for CMV. The patients included in this control group were selected according to the availability of specimens.
All sections studied were selected according to the presence of medium to large bile duct and hepatic artery tissue (the primary targets of chronic rejection).

The patients included in this study are described in Appendices 1 and 3.

All sections selected for study had been examined previously by an experienced pathologist; these reports were used throughout this study to define the histopathology of each section.

6.2.2 Preparation of Slides

Formalin fixed, paraffin embedded tissue sample slides were kindly provided by Dr Nick Coleman.

Formalin Fixation and Paraffin Embedding

Specimens were fixed by immersion in 10% formol saline for 24 hours. Tissue was then embedded using an automatic processor which immersed the specimen sequentially in absolute ethanol for 9 hours (changed after 2 hours then hourly), xylene for 3 hours (changed after 1.5 hours), molten paraffin wax (56°C) for 2 hours before embedding into blocks and cooling to room temperature.

Preparation of Tissue Sections

Slides were washed in distilled water (2 x 3 minutes), rinsed in industrial methylated spirits (IMS) and air dried. Slides were then soaked in 2% (v/v) aminopropyltri-ethoxysilane (APES) in dry acetone for 5 seconds and then in distilled water for 2 minutes before drying overnight at 37°C.
Sections were cut (4-5μm) using a new blade for each specimen and mounted onto the APES-coated slides before drying overnight at 37°C. This was performed the day before screening.

6.2.3 Immunohistochemistry

Antibodies

Four primary antibodies were used in this study; a monoclonal antibody against CMV delayed-early DNA binding protein p52 (raised in mouse; Dako), a monoclonal antibody raised against rubella which served as an irrelevant isotype-matched control, a monoclonal raised against cytokeratin to identify epithelial cells (raised in mouse; MNF116, Unipath) and a monoclonal antibody to identify endothelial cells (raised in mouse; Q. Bend 10, Unipath).

Goat-anti mouse (Dako) was used as secondary antibody; this was conjugated to biotin. Development was achieved by application of streptavidin/biotin complex conjugated to horse radish peroxidase (HRP).

The anti-endothelial and anti-epithelial antibodies were used to confirm putative epithelial and endothelial positivity for CMV. The manufacturers claim specificity; antibody to CMV p52 protein does not cross react with any other herpesvirus.

Automated Procedure for Immunohistochemistry

This technique was performed using an automated system that was set up in the Histopathology Department, Addenbrooke's Hospital.

Slides were dewaxed in xylene for 5 minutes and washed in water for 5 minutes before blocking of endogenous peroxidase activity (2.5% hydrogen peroxidase (v/v) in methanol) for 10 minutes. Slides were washed again in water for 6 minutes before trypsinisation (0.1% trypsin, 0.1% CaCl (pH7.8))
water for 6 minutes before trypsinisation (0.1% trypsin, 0.1% CaCl (pH7.8)) at 37°C for 20 minutes, a further wash in water for 3 minutes to stop enzyme activity and equilibration in Tris-buffered saline (TBS) for 1 minute. The primary antibody was then applied (diluted 1:500 in TBS) for 30 minutes before removal of non-specifically bound antibody by 2 x 10 minute washes in TBS. The secondary antibody-conjugate (diluted 1:1000 in TBS) was then applied for 30 minutes before washes to remove antibody bound non-specifically (2 x 10 minutes with TBS) and application of tertiary solution (streptavidin/biotin complex conjugated to HRP (1:100 in TBS); Dako) for 30 minutes and further washes (2 x 10 minutes with TBS). Development was achieved by addition of substrate/chromagen (0.05M Tris, 0.04M HCl, 2.5% (v/v) diaminobenzidine, 0.2% hydrogen peroxide) for 10 minutes. This reaction was stopped by washing in water for 10 minutes and slides were then counterstained in Harris' Haematoxylin for 15 seconds, rinsed in water, dried and mounted.

Immunohistochemistry was also performed without application of primary antibody for each slide processed; this served as a control for secondary antibodies and conjugate. A positive control (shown previously to be CMV infected) was also included in each run.

### 6.2.4 In Situ Hybridisation (ISH)

**Probe for CMV**

A digoxigenin-labelled 23kB probe was kindly supplied by Dr Fraser Lewis (University of Leeds, Leeds, UK); this comprised the Hind III F fragment from the genome of the CMV strain AD169. Labelling was performed by nick translation which generated labelled fragments of 300 - 500 base pairs. This probe showed minimal homology to cellular DNA (130, 173).
Control Probe

A control probe comprised mouse cellular DNA (balb/c strain) which was labelled with digoxigenin by nick translation according to manufacturers instructions (Boehringer Mannheim). Labelling was performed by nick translation which generated labelled fragments of 300 - 500 base pairs. All sections that were scored positive after in situ hybridisation with the CMV probe were also screened using the mouse DNA probe.

Prevention of RNA'se Contamination

This was achieved by wearing disposable gloves at all times, using sterile plastic ware and/or baked glassware (180°C for 8 hours). Water was treated with diethyl pyrocarbonate (DEPC); 500μl DEPC was mixed with 500ml water, incubated at room temperature overnight and then autoclaved.

ISH Method

Wash and immersion steps were carried out in Hellendahl jars. Slides were dewaxed in xylene (2 x 10 minutes) and rehydrated by serial immersion (for 2 minutes at room temperature) in 99%, 95%, 70% and 50% ethanol followed by immersion in water for 5 minutes at room temperature and then 2 x SSC (20X SSC stock; 3M NaCl, 0.3M trisodium citrate) for 10 minutes at 60°C.

Rehydrated slides were then processed using reagents from an ISH kit (R&D Systems) following the manufacturers instructions with modifications. Equilibration in 50mM Tris for 5 minutes was performed before 100μl proteinase K solution (50μg/ml in 50mM Tris) was pipetted onto each section which were then incubated in humidity chambers for 30 minutes at 37°C. Slides were then rinsed in phosphate buffered saline.
(PBS), fixed (0.4% paraformaldehyde in PBS for 20 minutes at 4°C) and rinsed in water.

Dehydration was now carried out before hybridisation; slides were serially immersed (for 2 minutes at room temperature) in 50%, 70%, 95% and 99% ethanol and air dried.

Hybridisation was carried out as follows. Probe was added to hybridisation solution containing 30% formamide (R&D Systems) to a final concentration of 200ng/ml. A coverslip was placed and sealed with rubber cement (dried after application at 65°C for 10 minutes) before both probe and tissue DNA were denatured by heating the slide at 80°C for 10 minutes. Slides were then placed in a 37°C incubator for 18 hours and hybridisation took place.

Coverslips and cement were then removed and probe bound non-specifically was removed by washing in 4 X SSC then 2 X SSC (both for 5 minutes at room temperature) before equilibration in modified Tris buffered saline (TBS)/Triton (R&D Systems) for 15 minutes at room temperature.

Detection of digoxigenin was then carried out using a kit supplied by Boehringer Mannheim following the manufacturer's instructions (see section 2.2.8 for method and buffers). Briefly, sections were equilibrated in buffer 1/ 0.3% Tween for 5 minutes and blocked with buffer 2 before application of anti-digoxigenin Fab fragment-alkaline phosphatase conjugate (1:500 in buffer 2) for 30 minutes at room temperature. Antibody bound non-specifically was removed by 3 x 15 minute washes with buffer 1/ 0.3% Tween at room temperature. Slides were then equilibrated in

250
buffer 3 for 5 minutes before development. Developing buffer comprised 0.34 mg/ml nitroblue tetrazolium (NBT)/ 0.18 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer 3.

Incubation was carried out overnight at room temperature. The reaction was stopped by washing in water for 3 minutes before counterstaining with haemotoxylin-eosin for 15 seconds. Sections were then air dried and mounted in glycerine.

ISH was also performed without addition of probe for each slide processed; this served as a control for the antibody-conjugate. A positive control (shown previously to be CMV infected by immunohistochemistry) was included in each run.

Sections were scored for the presence of a signal by a semiquantitative method ie. low, medium and high positivity, for each infected cell type. This was performed 'by eye' by myself and results were confirmed by an experienced pathologist (Dr Nick Coleman; Pathology, Addenbrookes NHS Trust).

6.2.5 CMV Antibody Status and Active CMV Infection
Each recipient/donor pair was tested for antibodies to CMV and each recipient was monitored regularly for active CMV infection by PCR, DEAFF and culture (these methods are described in Chapters 2 and 3).

6.2.6 HLA Typing
The HLA type of each recipient/donor pair was tested (methods are described in Chapter 4).
6.3 Results

6.3.1 Raw Data
The data analysed in this chapter are given fully for each patient studied in Appendix 3.

6.3.2 Cells Shown to Harbour Active CMV Infection
Active CMV infection was demonstrated in hepatocytes, infiltrating mononuclear cells, bile duct epithelial cells and endothelial cells of hepatic artery and portal vein.

6.3.3 In Situ Hybridisation Detection of CMV Occurred via DNA: DNA Hybridisation
In situ hybridisation was repeated for specimens in which CMV had been detected by this technique. Two reactions were set up for two serial sections from each specimen; one in which the specimen DNA was denatured before hybridisation and one in which this step was omitted. In situ hybridisation only occurred when specimen DNA was denatured before application of the probe. This showed that in situ hybridisation occurred by probe DNA: target DNA interaction (note that probe DNA: target RNA interaction does not require denaturation of target DNA).

6.3.4 Specificity of In Situ Hybridisation to CMV DNA
No signal was detected in the absence of probe; the digoxigenin detection system did not mediate false positivity.

Digoxigenin labelled mouse DNA was used against all specimens in which CMV had been detected by in situ hybridisation. This control probe did not hybridise to any of the sections tested (a positive control for this
experiment (mouse lung section) was positive by \textit{in situ} hybridisation.) These control experiments showed that the CMV probe hybridised specifically to CMV infected cells.

6.3.5 Distribution of CMV Positive Cells Amongst Liver Transplant Recipients According to CMV Antibody Status and Active CMV Infection

These data are summarised in table 6.1 and important points are described below.
Table 6.1 Distribution of CMV Positive Cells Amongst 29 Liver Transplant Recipients According to CMV Antibody Status and Active CMV Infection

<table>
<thead>
<tr>
<th>CMV SURVEILLANCE</th>
<th>Tissue positive (Immuno. or ISH)</th>
<th>Immunohistochemistry</th>
<th>In Situ Hybridisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>donor/recipient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/ - n=11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes n=11</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>No n=0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+/ + n=6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes n=2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No n=4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>-/ + n=4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes n=1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No n=3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-/ - n=4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes n=0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No n=4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>?/ + n=4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes n=2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No n=2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6.1 shows the number of patients that harboured active CMV in graft tissue by immunostaining or in situ hybridisation in relation to CMV antibody status and active CMV infection (Yes= active CMV infection post-transplant, No= active CMV-free). The number of patients in each group that had CMV detected in the graft, by immunostaining or in situ hybridisation, is given (Tissue positive (Immuno. or ISH)). The results generated by immunostaining or in situ hybridisation are also shown separately; for both methods, the number of patients in whom CMV was shown to infect various cells is shown (Hep.= hepatocytes, Mono.= mononuclear cells, Epi.= epithelial cells and End.= endothelial cells) together with the number of patients that had CMV detected in the graft irrespective of cell type (Tissue).

The results shown in table 6.1 show that most of the patients (6/11) that harboured active CMV in the graft were CMV antibody negative pre transplant and received a graft from a CMV antibody positive donor; all of the patients with this CMV antibody pattern were shown to develop active
CMV infection by viral surveillance of serum and/or buffy coat and/or urine.

However, two CMV antibody positive recipients were shown to harbour active CMV in the graft without detection of active CMV by viral surveillance of serum and/or buffy coat and/or urine (patients 8 and 12).

Furthermore, two CMV antibody negative recipients that received grafts from CMV antibody negative donors and were consistently negative for active CMV after transplantation by viral surveillance of serum and/or buffy coat and/or urine, were shown to harbour active CMV in the graft (patients 11 and 15).

All of the sections and cell types for each section, that were shown to be positive for CMV by immunohistochemistry were also shown to be positive for CMV by in situ hybridisation but the converse was not true; in situ hybridisation was more sensitive than immunohistochemistry. With respect to the number of sections recorded as positive, the sensitivity of immunohistochemistry in relation to in situ hybridisation was 64% (7/11).

Summary

- Liver graft vascular endothelial cells and bile duct epithelial cells were shown to harbour actively replicating CMV; this was a novel discovery.

- CMV was detected in liver in a high proportion of CMV antibody negative recipients that received grafts from CMV antibody positive donors; each of these also developed active CMV infection detected by viral surveillance of serum and/or buffy coat and/or urine.
Furthermore, CMV DNA in liver was detected for two recipients that were CMV antibody negative and received grafts from CMV antibody negative donors.

In situ hybridisation was more sensitive than immunohistochemistry; all sections (and cell types for each section) that were positive for active CMV infection by immunohistochemistry were also positive by in situ hybridisation but the converse was not true.

6.3.6 Distribution of CMV Positive Cells Amongst Liver Transplant Recipients with the Development of Chronic Rejection

The results shown here were generated exclusively by in situ hybridisation; the results generated by immunohistochemistry are unhelpful because this technique lacks sensitivity (see above).

Overall, 41% (12/29) patients studied harboured active CMV in the liver graft; these patients comprised 50% (4/8) of patients that developed chronic rejection, 50% (2/4) of patients that developed hepatic artery thrombosis, 100% (3/3) of patients with grafts lost to other diseases and 29% (4/14) of patients that retained grafts.

Active CMV infection was detected by in situ hybridisation (ISH) and immunohistochemistry in hepatocytes, mononuclear cells, bile duct epithelial cells and endothelial cells of hepatic artery and portal vein.

The distribution of CMV-positive cells amongst patients that developed chronic rejection (CR) or hepatic artery thrombosis (HAT) and controls is given in table 6.2. Note that the control population was biased towards patients at risk of active CMV infection of the graft (see section 6.2.1) and comprised 10 patients that developed active CMV infection (detected by viral surveillance of serum and/or buffy coat and/or urine) after transplantation and four patients that did not.
Table 6.2 shows the distribution of CMV-positive hepatocytes, mononuclear cells, epithelial cells and endothelial cells amongst patients that developed chronic rejection ('CR'), patients that developed hepatic artery thrombosis ('HAT'), patients with graft loss to other diseases ('other loss') and those who retained grafts ('retained'). The total number of patients that harboured active CMV in the graft is shown for each group (total graft positive). The proportion of CMV positive grafts (total graft positive) and CMV positive cell types (columns) were compared between chronic rejectors (CR) and controls ('other loss' + 'retained') and between patients that developed HAT and controls ('other loss' + 'retained') using Fisher's exact test (p values given).

CMV positive hepatocytes and mononuclear cells were found in 10 and 7 recipients respectively and were equally prevalent amongst patients that developed chronic rejection, hepatic artery thrombosis or controls. In contrast, epithelial cell positivity was higher amongst patients that developed chronic rejection; however this result was not significant. Endothelial cell positivity was a relatively rare event but was seen for four patients; none of these lost the graft to chronic rejection and two patients retained the graft.
Summary

- Overall, CMV was detected in liver at similar rates between patients with chronic rejection (CR) or hepatic artery thrombosis (HAT) and a control population ('other loss' plus 'retained') heavily biased towards patients with active CMV infection.

- Bile duct epithelial cell infection by CMV was higher amongst patients that lost the liver graft to chronic rejection. However, this result was not significant.

6.3.7 HLA Matching/ Mismatching Between Recipient and Donor and Active CMV Infection of the Graft

Results presented in Chapter 4 suggested that HLA class I mismatching was a risk factor for persistent active CMV infection and it was suggested that this result reflected persistent infection of the graft; this was tested directly and results are given in table 6.3.

Table 6.3 Distribution of Mismatched HLA Alleles for Liver Transplant Recipients With or Without Active CMV Infection of the Graft as Detected by In Situ Hybridisation

<table>
<thead>
<tr>
<th>Mismatches</th>
<th>HLA A</th>
<th>HLA B</th>
<th>HLA A or B</th>
<th>HLA A and B</th>
<th>HLA DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV positive graft (n=12)</td>
<td>50%</td>
<td>83%</td>
<td>83%</td>
<td>50%</td>
<td>42%</td>
</tr>
<tr>
<td>CMV negative graft (n=17)</td>
<td>53%</td>
<td>59%</td>
<td>76%</td>
<td>35%</td>
<td>41%</td>
</tr>
</tbody>
</table>

p= 0.29 p=0.13 p=0.33 p=0.22 p=0.30

Table 6.3 shows the proportion of patients that were shown to develop active CMV infection of the graft (CMV positive graft) and those whose graft did not develop active CMV infection (CMV negative graft) according to mismatches for HLA alleles A, B and DR. Results were generated by in situ hybridisation and statistical analysis was performed using Fishers exact test.

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The data shown in table 6.3 were not conclusive. Detection of CMV DNA in liver was associated with an increased proportion of HLA B mismatched donor/recipient pairs. This result supported the association between HLA B mismatching and prolonged active CMV infection as detected by viral surveillance of serum and/or buffy coat and/or urine (see Chapter 4); however, the numbers tested were small and this result was not significant.

Summary

- HLA B allele mismatching occurred in a higher proportion of grafts that were positive for active CMV infection but this result was not significant.

6.3.8 The Significance of Active CMV Infection of Liver Graft Epithelial or Endothelial Cells

Table 6.2 showed that epithelial cell positivity was higher amongst patients that developed chronic rejection and it is possible that active CMV infection of epithelial cells is particularly important in this setting.

In situ hybridisation results, infiltration and HLA typing data were compared for each patient that was shown to harbour CMV in epithelial or endothelial tissue and are shown in table 6.4; these patients are numbered according to the system used in Appendix 1.
Table 6.4 Patients Harbouring Active CMV in Epithelial and Endothelial Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>CMV Ab.</th>
<th>Graft loss</th>
<th>Active CMV</th>
<th>CMV positive cells</th>
<th>HLA matching</th>
<th>Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>?/+</td>
<td>Y</td>
<td>CR</td>
<td>+</td>
<td>- - +(L)</td>
<td>0 0 0</td>
</tr>
<tr>
<td>3</td>
<td>+/-</td>
<td>Y</td>
<td>CR</td>
<td>+(L) + (L) + (L)</td>
<td>-</td>
<td>0 0 1</td>
</tr>
<tr>
<td>8</td>
<td>+/-</td>
<td>Y</td>
<td>'other loss'</td>
<td>+(M) + (H) + (L)</td>
<td>+(L)</td>
<td>1 0 0</td>
</tr>
<tr>
<td>12</td>
<td>+/-</td>
<td>Y</td>
<td>CR</td>
<td></td>
<td>- - +(M)</td>
<td>1 1 0</td>
</tr>
<tr>
<td>29</td>
<td>+/-</td>
<td>Y</td>
<td>HAT</td>
<td>+(H) + (H) + (L)</td>
<td>+(L)</td>
<td>0 0 1</td>
</tr>
<tr>
<td>26</td>
<td>+/-</td>
<td>N</td>
<td>----</td>
<td>+(H) + (H) + (L)</td>
<td>+(L)</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

Table 6.4 shows features of 6 patients that harboured active CMV in endothelial and/or epithelial cells. The CMV antibody status (CMV Ab.) of donor/recipient (D/R) is shown alongside the occurrence of active CMV infection as detected by viral surveillance of serum and/or buffy coat and/or urine (Active CMV). CR= chronic rejection, HAT= hepatic artery thrombosis. Cells that harboured active CMV are indicated as Hep.= hepatocyte, Mono.= mononuclear cell, Epi.= epithelial cell and End.= endothelial cell and the quantity of positive cells found is indicated as low (L), moderate (M) or high (H). Infiltrating mononuclear cells were defined as follows: LD= low diffuse and LP, MP and HP= low, moderate and high aggregation around portal tracts respectively. Note that all chronic rejectors also had infiltration of foam cells (macrophages) into the lumen of hepatic arteries.

Only one patient that retained the liver graft (patient 26) had active CMV infection of epithelial and endothelial cells; this patient died of CMV disease. One other control (patient 8) had active CMV infection of epithelial and endothelial cells; graft loss occurred through hepatitis C related cirrhosis.

A number of important points can be extracted from this data; these are summarised below.
Summary

- Epithelial CMV infection was observed for two patients without detection of active CMV by viral surveillance of serum and/or buffy coat and/or urine.

- Epithelial and endothelial CMV infection was observed in one graft that was retained (patient 26); this patient died of CMV disease.

- Epithelial and endothelial CMV infection was observed in one graft that was lost to hepatitis C related cirrhosis (patient 8).

- Epithelial CMV infection was associated with moderate to high mononuclear cell infiltration of the portal tract for two patients (patients 1 and 8).

- In contrast, endothelial CMV infection was observed only in grafts that contained high numbers of infected mononuclear cells and, in 2/3 cases, hepatocytes.

- However, there was no obvious relationship between graft loss to chronic rejection or hepatic artery thrombosis, active CMV infection of epithelial cells or endothelial cells and HLA allele matching status.

6.3.2 Cases

Several cases are shown; fuller descriptions of each patient can be found in Appendix 1 (patient numbers given here). The data presented comprises my own observations of immunohistochemistry and \textit{in situ} hybridisation results together with retrospective pathology reports of each section by Dr Nick Coleman and Dr Derek Wight.

In the figures shown, CMV infected cells detected by immunohistochemistry are brown in colour whereas those detected by \textit{in situ} hybridisation are purple in colour; CMV infected cells are indicated by arrows. Structures are labelled as follows: PT= portal tract, BD= bile duct, VBD= 'vanished' bile duct, HA= hepatic artery, PV= portal vein, FM= foamy macrophages, H=hepatocyte, M= mononuclear cell, Epi= epithelial cell, Endo= endothelial cell.
Patient 1

This patient developed active CMV infection post transplant before development of chronic rejection and subsequent loss of the liver graft; the section shown was taken from the explanted 'chronically rejected' liver (see also Appendixes 1 and 3).

The histopathology of the section studied was characteristic of chronic rejection. Bile ducts were absent from most portal tracts which contained moderate numbers of inflammatory mononuclear cells and pericellular fibrosis was observed around bile ducts. Furthermore, many medium and small sized arteries contained foam cells in the lumen.

Figure 6.2 shows a typical portal tract from this section which was stained for CMV DNA by in situ hybridisation. The lumen of the hepatic artery (HA) shown was occluded by foamy macrophages (FM) and the ghost of a bile duct could be identified by its position in the portal tract and the configuration of surrounding inflammatory cells. This photograph was also shown in figure 6.1 (see section 6.1) to contrast with the appearance of a normal portal tract.

Foamy macrophages (FM) and mononuclear cells (M) were not positive for CMV DNA. This result was consistent for all portal tracts examined in this case that displayed this histopathology and indeed was consistent for all cases of chronic rejection studied.
In figure 6.2 a portal tract (PT) is shown comprising 'vanished' bile duct (VBD), hepatic artery (HA) with occlusion of the lumen by foamy macrophages (FM) and portal vein (PV). This photograph was taken at magnification x200. Active CMV infection was not observed.

In this case, CMV DNA was not detected in 'vanished' bile ducts or occluded hepatic arteries that displayed features characteristic of chronic rejection. However, epithelial cells of numerous small and medium sized bile ducts that did not display the characteristic histopathology of chronic rejection were positive for active CMV.

Figures 6.3 (magnification x200) and 6.4 (magnification x400) show a typical bile duct from this case that contained bile duct epithelial cells that were actively infected with CMV which was detected predominantly in the cytoplasm of these cells.
Figure 6.3  Active CMV Infection of Bile Duct Epithelial Cells Shown by In Situ Hybridisation (Patient 1)

Figure 6.3 shows bile ducts (arrows) which have active CMV infection of epithelial cells (arrowheads). This photograph was taken at magnification x200.

Figure 6.4  Active CMV Infection of Bile Duct Epithelial Cells Shown by In Situ Hybridisation (Patient 1)

Figure 6.4 also shows bile ducts (arrows) which have active CMV infection of epithelial cells (arrowheads) but at higher magnification (x400).
Moderate numbers of mononuclear cells were often observed to surround CMV infected epithelial cells (see figure 6.5; x 200 magnification).

Figure 6.5  Active CMV Infection of Bile Duct Epithelial Cells Shown by In Situ Hybridisation and Aggregation of Mononuclear cells (Patient 1)

Figure 6.5 shows a bile duct (BD) with active CMV infection of epithelial cells (arrowheads); this bile duct was surrounded by infiltrating mononuclear cells (M). This photograph was taken at magnification x400.

Patient 8

This patient also developed active CMV infection post transplant before subsequent loss of the liver graft to hepatitis C related cirrhosis. The section shown was taken from the explanted liver (see also Appendixes 1 and 3).
Portal vein endothelial cells and bile duct epithelial cells were shown to be actively infected with CMV; hepatocytes and mononuclear cells were also positive for active CMV.

Figure 6.6 shows two medium sized portal veins; one contains a CMV-infected endothelial cell and the other is in close proximity to two CMV-infected mononuclear cells. These are shown at higher magnification in figures 6.7 and 6.8.

**Figure 6.6**  **Active CMV Infection of Portal Vein Endothelial Cells and Mononuclear Cells Shown by In Situ Hybridisation (Patient 8):**

Figure 6.6 shows two portal veins (arrows); one contained a CMV-infected endothelial cell (arrowhead) and the other was in close proximity to two CMV-infected mononuclear cells (arrowheads). This photograph was taken at magnification x100.
Figure 6.7  Active CMV Infection of a Portal Vein Endothelial Cell Shown by In Situ Hybridisation (Patient 8)

Figure 6.7 shows a portal vein (arrow) that contained a CMV-infected endothelial cell (arrowhead) at magnification x200.

Figure 6.8  Active CMV Infection of Two Mononuclear Cells in Close Proximity to a Portal Vein Shown by In Situ Hybridisation (Patient 8)

Figure 6.8 shows a portal vein (arrow) which was in close proximity to two CMV-infected mononuclear cells (arrowheads). This photograph was taken at magnification x200.
Patient 12

This patient lost the liver graft to chronic rejection; the section shown was taken from the explanted 'chronically rejected' liver. This patient was CMV antibody positive before transplantation and received a graft from a CMV antibody positive donor. However, active CMV infection was not detected post transplant by viral surveillance of serum and/or buffy coat and/or urine (see also Appendixes 1 and 3).

The histopathology of the section studied was characteristic of chronic rejection. Bile ducts were absent from numerous portal tracts and many medium and small sized arteries contained foam cells in the lumen. A low level, diffuse infiltration of mononuclear cells was observed without aggregation around portal tracts.

Figure 6.9 shows a medium sized hepatic artery from this section which was stained for CMV DNA by in situ hybridisation. The lumen of this hepatic artery (HA) was occluded by foamy macrophages (FM) which were not positive for CMV DNA. This result was consistent for all portal tracts examined in this case that displayed histopathology characteristic of chronic rejection.
In figure 6.9 a hepatic artery (HA) with occlusion of the lumen by foamy macrophages (FM) is shown. This photograph was taken at magnification x200.

However, bile duct epithelial cells of other, apparently undiseased, portal tracts were shown to be actively infected with CMV; hepatocytes and mononuclear cells were also positive for active CMV.

Epithelial cells of one large bile duct and several small bile ducts were positive for active CMV in this case. These are shown in figure 6.10 and at higher magnification in figure 6.11. Most of the epithelial cells of these bile ducts were actively infected by CMV; positivity was observed for both cytoplasm and nuclei of these cells. Very little inflammation was observed.
Figure 6.10  Active CMV Infection of Bile Duct Epithelial Cells Shown by In Situ Hybridisation (Patient 12)

Figure 6.10 shows bile ducts (arrows) with active CMV infection of epithelial cells (arrowheads). This photograph was taken at magnification x200.

Figure 6.11  Active CMV Infection of Bile Duct Epithelial Cells Shown by In Situ Hybridisation (Patient 12)

Figure 6.11 shows bile ducts (arrows) with active CMV infection of epithelial cells (arrowheads). This photograph was taken at magnification x400.
Patient 29

This patient died from hepatic artery thrombosis on day 204 after transplantation; the section shown was taken from a needle biopsy which was performed on day 41 for suspected CMV hepatitis which was confirmed. Active CMV infection was also detected from day 45 to day 155 post transplant by viral surveillance of serum and/or buffy coat and/or urine (see also Appendixes 1 and 3).

The histopathology of the section studied was characteristic of CMV hepatitis; there was no evidence of rejection. Large numbers of CMV-infected hepatocytes and mononuclear cells were shown by immunohistochemistry and in situ hybridisation; in addition, active CMV infection of two bile duct epithelial cells (see figures 6.12 and 6.13) and one hepatic artery endothelial cell (see figure 6.14) was shown. Little diffuse inflammation was seen but there was some low level aggregation of mononuclear cells around portal tracts.
Figure 6.12  Active CMV Infection of Bile Duct Epithelium Shown by Immunohistochemistry (Patient 29)

Figure 6.12 shows active CMV infection of two bile duct epithelial cells (arrowheads); these cells were confirmed to be epithelial cells as shown in figure 6.13. This photograph was taken at magnification x200.

Figure 6.13  Bile Duct Epithelium Shown by Immunohistochemistry (Patient 29)

Figure 6.13 shows detection of epithelial cells (arrows) by immunohistochemistry for cytokeratin. This photograph was taken at magnification x200. The slide stained was adjacent to that shown in figure 6.12 (stained for CMV) and confirmed active CMV infection of bile duct epithelial cells.
Summary

- Active CMV infection was observed in a graft lost to hepatitis C related cirrhosis.

- In patients that developed chronic rejection, active CMV infection of bile duct epithelial cells or vascular endothelial cells was not demonstrated in portal tracts that displayed the histopathology of chronic rejection ie. absence of bile ducts and occlusion of hepatic artery lumen with foam cells.

- However, active CMV infection of bile duct epithelial cells or vascular endothelial cells was demonstrated in adjacent portal tracts of normal appearance.
6.4 Discussion

The results presented are based upon a small highly selected group of patients and must be interpreted with caution. However, these results are provocative and allow a number of hypotheses to be set up.

6.4.1 Conclusions

The Hypothesis Tested

- The hypothesis that CMV infection of bile duct epithelial and/or hepatic artery endothelial cells initiates or enhances chronic rejection was upheld but not proven.

Active CMV Infection of the Liver Graft and CMV Antibody Status

- CMV antibody negative recipients that receive grafts from CMV antibody positive donors and subsequently develop active CMV infection (detected by viral surveillance of serum and/or buffy coat and/or urine) may be at high risk of active CMV infection of the liver graft.

- However, active CMV infection of the liver graft may also occur without detection of active CMV infection by viral surveillance of serum and/or buffy coat and/or urine.

- Furthermore, active CMV infection of the liver graft may also occur in CMV antibody negative recipients that receive grafts from CMV antibody negative donors.

Active CMV Infection of the Liver Graft and HLA Status

- Mismatching for the HLA B allele may be associated with an increased risk of active CMV infection of the liver graft as predicted in Chapter 4; however, this result was not significant and a firm conclusion cannot be drawn.

Active CMV Infection of the Liver Graft and Chronic Rejection

- Active CMV infection of bile duct epithelial cells and hepatic artery endothelial cells occurs; these sites are the primary sites of damage for chronic rejection.

- Active CMV infection of bile duct epithelial cells may be associated with CR; only two control patients had active CMV infection of epithelial cells and these
patients died of CMV disease or were retransplanted for hepatitis C related cirrhosis respectively.

• In sections taken from liver grafts that were explanted after chronic rejection, active CMV infection of bile duct epithelial cells or vascular endothelial cells was only demonstrated in portal tracts of normal appearance and was not demonstrated in adjacent portal tracts that were obviously damaged; it is possible that active CMV infection of these sites is an important event during the early stages of development of chronic rejection and that cells harbouring active CMV are destroyed.

6.4.2 In Situ Hybridisation was a Specific and Sensitive Technique

In situ hybridisation using a probe comprising the Hind III F fragment from the genome of the CMV strain AD169 identified numerous cells that contained replicating CMV. Hybridisation occurred via DNA: DNA interaction; hybridisation did not occur when target nucleic acids were not heat denatured. In a series of similar experiments, DNA was also shown to be the target nucleic acid by the Kings College group (423, 424).

The specificity of this probe was confirmed. Sections that were positive were also screened using digoxigenin labelled whole genomic mouse DNA (a nonhomologous control) and were also processed without the addition of any probe; positive signal against the human sections of interest was not generated by either control experiment. The specificity observed was expected because the Hind III F fragment has previously been shown to share minimal homology to cellular DNA (130, 173). In situ hybridisation was more sensitive than immunohistochemistry; this was also shown previously by the Kings College group (Naoumov et al (1988) (423)). Early antigen is produced throughout the replication cycle of CMV but it is possible that DNA replication exceeds early antigen production at late stages of this cycle.
6.4.3 Detection of Active CMV Infection of the Graft, CMV Antibody Status and Active CMV Infection as Detected by Viral Surveillance of Serum and/or Buffy Coat and/or Urine.

In situ hybridisation detected active CMV infection of grafts for four patients that were consistently negative for active CMV by viral surveillance of serum and/or buffy coat and/or urine. Furthermore, two of these patients were CMV antibody negative recipients of liver grafts from CMV antibody negative donors.

The latter result is consistent of the finding by Taylor-Weideman et al (1991) (41) that CMV antibody negative individuals may harbour latent CMV in monocytes (also see Chapter 1).

These results have important implications for clinical management of liver transplant recipients. It is important to differentiate between acute rejection and active CMV infection after liver transplantation; these two conditions are associated with similar symptoms and accurate diagnosis of active CMV infection is important (see Chapters 2 and 3).

Acute rejection is treated by administration of steroids and/or increasing immunosuppression whereas active CMV infection is treated by administration of antivirals and lowering of immunosuppression. Misdiagnosis of acute rejection for a patient with active CMV infection may therefore lead to administration of drugs that enhance CMV replication. Conversely, misdiagnosis of active CMV infection for a patient with acute rejection may therefore lead to a reduction of immunosuppression.
Patient 11 developed an unexplained hepatitis on day 98 after transplantation. This patient was CMV antibody negative and received a graft from a CMV antibody negative donor and was consistently negative for active CMV by viral surveillance of serum and/or buffy coat and/or urine. Furthermore, CMV was not detected in a liver biopsy taken at day 98 post transplant by either light microscopy or immunohistochemistry. Therefore, this patient was not treated with antivirals. The liver graft was lost to chronic rejection on day 129 and CMV was detected retrospectively in the explanted graft by \textit{in situ} hybridisation. It is likely that this patient had persistent active CMV infection which was localised to the liver graft.

Similarly, patient 12 developed unexplained hepatitis on day 16 and pyrexia on day 30 after transplantation. This patient was CMV antibody positive and received a graft from a CMV antibody positive donor but was consistently negative for active CMV by viral surveillance of serum and/or buffy coat and/or urine. This patient was treated with ganciclovir on day 32 for 14 days despite the lack of confirmation of active CMV by laboratory testing. The liver graft was lost to chronic rejection on day 289 and CMV was detected retrospectively in the explanted graft by \textit{in situ} hybridisation. It is likely that this patient also had persistent active CMV infection which was localised to the liver graft.

I therefore recommend that all liver graft biopsies taken after transplantation are stained for CMV DNA using \textit{in situ} hybridisation regardless of the CMV antibody status or 'active CMV status' of the patient. This policy may enable diagnosis of localised active CMV infections of the liver graft where the sensitivity of light microscopy or immunohistochemistry are insufficient.
Furthermore, if active CMV infection of the graft is important in the development of chronic rejection then it is possible that the lack of association between active CMV infection as detected by viral surveillance of serum and/or buffy coat and/or urine or CMV antibody status and chronic rejection as reported in this thesis (see Chapter 4) and by others (372, 225, 210) is because patients with localised active CMV infection of the graft (including those donor/recipient pairs that are CMV antibody negative) are excluded from such analyses.

6.4.4 Active CMV Infection of the Liver Graft and Chronic Rejection

Two groups have shown that active CMV of the liver graft is associated with chronic rejection (225, 284). However, liver graft recipients that develop active CMV infection of the graft do not necessarily develop chronic rejection and therefore other factors must be involved. The study presented in this chapter was designed to compare patients that developed chronic rejection and those that developed active CMV infection of the graft but not chronic rejection; therefore, the control group was biased towards patients in whom active CMV infection had been detected by viral surveillance of serum and/or buffy coat and/or urine, i.e. those at high risk of active CMV infection of the graft.

The study reported by Arnold et al (1992) (284) showed that persistence of active CMV infection may be an important factor for development of chronic rejection. The results presented in this thesis suggest that the type of cells (i.e. epithelial cells) that are actively infected with CMV may also be an important factor that discriminates between those that develop chronic rejection and those that do not.
Interestingly, active CMV infection of the graft was seen concomitantly with hepatitis C related cirrhosis for patient 8 who lost the graft to the latter disease. Pre transplant diagnosis of hepatitis C may be a risk factor for chronic rejection (see section 4.3.4, 427, 428). It is possible that in some cases hepatitis C virus and CMV act in concert to elicit chronic rejection. Alternatively, hepatitis C virus may facilitate CMV infection which in turn may elicit chronic rejection. This is supported by the findings of Singh et al (1996) (429) who showed a significantly higher rate of CMV disease in liver transplant patients with recurrent hepatitis C.

6.4.5 Persistence Active CMV Infection, Cellular Immunity and Chronic Rejection

The Kings College group (224, 284; see sections 1.8.2 and 1.9.1) suggested that complete mismatching of HLA class I alleles acted indirectly as a risk factor for chronic rejection by preventing immune surveillance by CMV-specific T-lymphocytes and therefore facilitating persistent active CMV infection of the graft.

Furthermore, matching of HLA DR alleles was shown to be a risk factor for chronic rejection and it was hypothesised that this facilitated MHC restricted presentation of CMV peptides or 'foreign' MHC class I peptides and therefore facilitated cellular immune mediated lysis of graft cells.

6.4.5.1 Detection of Active CMV Infection in Bile Duct Epithelial Cells and Hepatic Artery Endothelial Cells

The results presented in this thesis showed that active CMV infection of bile duct epithelial cells and hepatic artery endothelial cells may occur after liver transplantation. In contrast, Arnold et al (1992) (284) observed CMV infection of hepatocytes but not of bile duct epithelial cells or hepatic artery endothelial cells after screening biopsy specimens by in situ
hybridisation. The apparent confinement of active CMV infection to hepatocytes suggested that immune mediated lysis of bile duct or hepatic artery cells did not occur after presentation of CMV peptides by HLA DR (unless target cells were 'burnt out'). It was therefore suggested that presentation of CMV peptides by hepatocytes may indirectly lead to damage of adjacent bile duct or hepatic artery tissue via local upregulation of cytokines and/or adhesion molecules thus enhancing the activity of alloreactive T-lymphocytes (see sections 1.9.3 and 1.9.4).

However, the novel finding presented in this thesis of active CMV infection of vascular endothelium and bile duct epithelium provides evidence that CMV may be directly involved with chronic rejection. One possibility is that CMV is cytopathic. Another possibility is that HLA DR presentation of CMV peptides leads directly to immune mediated lysis of these cells. It is likely that CMV infection of the latter two cell types was observed in this thesis, but not by Arnold et al (1992) (284), because sections of these tissues were selected from explanted organs in preference to biopsy specimens.

Active CMV infection of bile duct epithelial cells may be important; this was found for 3/8 patients that developed chronic rejection and for only 2/17 control patients. Patient 26 died from CMV disease and patient 8 lost the graft to hepatitis C related cirrhosis thus preventing long-term follow up for chronic rejection.

These three chronic rejectors only harboured active CMV in apparently undamaged portal tracts; adjacent portal tracts displaying the characteristic histopathology of chronic rejection were CMV free. This suggests that CMV may be involved with the early stages of chronic rejection, possibly
as an inducer. The process of chronic rejection leads to loss of CMV permissive tissue and CMV may be cleared from damaged areas by phagocytes.

6.4.5.2 Persistence of Active CMV Infection in the Graft and Complete Mismatching of HLA Class I Alleles

The results presented in Chapter 4 of this thesis suggested that complete mismatching of class I alleles was associated with active CMV infections of long duration (>30 days) and it was suggested that such infections were a marker for persistent active CMV infection of the graft.

The effect of complete mismatching of HLA class I upon active CMV infection of the graft was examined here using in situ hybridisation. The results presented showed that complete mismatching of HLA B alleles was associated with a greater proportion of active CMV-positive grafts compared with partial or complete matching of HLA B alleles; however, this result was not significant. This result suggests that, for at least some liver graft recipients, complete mismatching for HLA B alleles was a risk for persistent active CMV infection of the graft.

It can thus be envisaged that CMV spread may occur unchecked in hepatocytes, which predominantly express HLA class I, but that subsequent infection of HLA DR-expressing endothelial and/or epithelial cells may trigger successful immune surveillance which leads to destruction of bile ducts and/or hepatic arteries.

6.4.5.3 Persistence of Active CMV Infection in the Graft, Matching of HLA DR Alleles and Chronic Rejection

Partial or complete matching of HLA DR alleles was an important component of the hypothesis put forward by the Kings College group. The results presented in this thesis showed that matching of HLA DR was
equally distributed between chronic rejectors and controls. However, this lack of association may have been due to the relatively low numbers of chronic rejectors studied. Furthermore, it is possible that there is more than one mechanism responsible for chronic rejection.

Three patients studied in this chapter (numbers 3, 29 and 26) fit this hypothesis perfectly. All 3 had active CMV infection of endothelial and epithelial cells and zero matched HLA class I. Patients 3 and 29 also received HLA DR matched grafts which they lost to CR and HAT respectively. In contrast, patient 26 received a zero HLA DR matched graft which was retained.

HLA DR upregulation has been shown during chronic rejection (191) which may enhance this process.

However, chronic rejection was also observed in patients that received grafts that were HLA class I matched (patients 8 and 12) and/or HLA class II zero matched (patients 1, 8 and 12). Therefore, other mechanisms must be operating in these grafts that do not involve the cellular immune system; alternative hypotheses are given below.

### 6.4.6 Active CMV Infection may Initiate Ischaemia

An attractive alternative hypothesis can be put forward which is analogous to accelerated atherosclerosis after cardiac transplantation (see section 1.8.3.3). A herpesvirus (Mareks disease virus) has been proven to mediate atherosclerosis in chickens (392) and there is evidence to suggest the involvement of CMV in the human form of the disease.
Ischaemic injury during the early stages of chronic rejection of human liver grafts has been described (191); these diseased arteries are histologically similar to atherosclerotic tissue. Furthermore, chronic rejection is often observed with little infiltration of mononuclear cells (191, 193); this is supported by the findings herein (patients 3, 12, 26 and 29).

It is possible that a process similar to atherosclerosis initiates endothelial damage which in turn initiates the clotting cascade that leads to development of hepatic artery thrombosis. It is also possible that CMV is involved with the development of hepatic artery thrombosis.

It has been hypothesised (388) that CMV-mediated damage to cardiac graft endothelial cells leads to damage of exposed smooth cells. A repair process induces proliferation of smooth muscle cells which leads to thickening of the intima and therefore occlusion of the lumen. A key piece of evidence for this hypothesis was the discovery of active CMV infection of coronary artery endothelial cells (385).

CMV infection of hepatic artery endothelial cells was not shown for any of the chronic rejectors studied. However, the demonstration of active CMV infection in hepatic artery endothelial cells of liver grafts allows this hypothesis to be applied to chronic rejection of liver grafts.

Endothelial cell positivity was shown for patient 29 who developed hepatic artery thrombosis which may be closely related to atherosclerosis.

Two control patients in this study (patients 8 and 26) hosted active CMV infection in hepatic artery endothelial cells. Patient 26 died from CMV disease and patient 8 lost the graft to hepatitis C related cirrhosis thus
preventing long-term follow up for chronic rejection or hepatic artery thrombosis.

6.4.7 Lytic Infection of Endothelial or Epithelial Cells
It is possible that CMV-mediated lysis of epithelial and/or endothelial cells leads to the damage of bile duct and/or hepatic artery; this also would explain the lack of HLA matching and infiltration observed in some chronically rejected grafts.

Epithelial cells have been shown to be fully permissive to CMV in vitro (251, 280; see section 1.8.2.3.2). This theory may explain the finding of 2 patients (1 and 12) that only harboured active CMV in bile duct epithelial cells (and are not matched for HLA DR).

The lack of positive mononuclear cells may be due to 'sampling error' but it is also possible that bile duct epithelial cells are a site of latency. Certainly, epidemiological evidence suggests that liver grafts harbour latent CMV (43).

6.4.8 Chronically Rejected Liver Grafts may Facilitate Active CMV Infection
It is possible that upregulation of HLA expression facilitates CMV infection and that the observed association between active CMV infection after transplantation and elevated HLA levels are due to opportunism of the virus during the initial sub-clinical stages of rejection. It has been suggested that the MHC molecule is a receptor for CMV (61, 62; see section 1.3.5).

Chronic rejection is associated with infiltration of foamy macrophages; monocyte-derived macrophages have been shown to be fully permissive
to CMV *in vitro* (41, 42, 48, 49; see sections 1.3.2 and 4.4.4). However, detection of active CMV infection of foamy macrophages was not shown in this thesis; it is possible that these infiltrating cells were not fully permissive to CMV as suggested by *in vitro* experiments.

Furthermore, production of inflammatory molecules such as interleukins and tumour necrosis factor-α may have a stimulatory effect upon CMV; TNF-α production and CMV replication have been shown to autoregulate each other positively (398-402; see Chapter 4).

Alternatively, CMV may lie latently in graft cells until the onset of chronic rejection; murine CMV has recently been shown to infect liver endothelial cells latently (44; see section 1.3.2) but this has not been confirmed for human CMV. Activation of CMV in a graft rejected chronically may occur, for example, after administration of steroids or differentiation of cells infected latently (48; see section 1.3.2).
CHAPTER 7

HUMAN HERPES VIRUS - 6 (HHV-6) IS NOT AN IMPORTANT PATHOGEN AFTER LIVER TRANSPLANTATION
7.1 Introduction

A number of reports document the importance of human herpes virus-6 (HHV-6) after bone marrow (354, 358, 359, 361) or renal (350, 355, 357, 364) transplantation. Furthermore, there is evidence to suggest that HHV-6 is associated with acute graft versus host disease after bone marrow transplantation (359, 361; see section 1.10.4.2) and acute rejection of renal grafts (350, 364).

In a series of 21 renal transplant patients, Okuno et al 1990 (350) found that 38% (8/21) developed active HHV-6 infection; all HHV-6-positive patients experienced severe acute rejection. Furthermore, HHV-6 antigens were detected by immunohistochemistry in acutely rejected tissue; tubular epithelial cells and infiltrating lymphocytes stained positive. A study of 72 renal transplant patients (364) found that a greater proportion of acutely rejected grafts contained HHV-6 antigens.

To my knowledge, only two reports have documented the incidence of active HHV-6 infection in liver transplant recipients (346, 423; see section 1.10.4.1).

Hypothesis

- Human herpes virus 6 (HHV-6) may be an important pathogen after liver transplantation and active infection may initiate or enhance chronic rejection.
7.2 Patients, Materials and Methods

7.2.1 Patients

Thirty three liver transplant and 17 bone marrow transplant recipients were selected according to the criteria given in sections 2.2.6 and 4.2.1. Bone marrow transplant patients were included in this study in an attempt to validate the polymerase chain reaction (PCR) test used for HHV-6; the incidence of active HHV-6 infection after bone marrow transplantation has been relatively well studied.

The bone marrow transplant group was chosen sequentially according to transplant date (see Appendix 2) whereas the liver transplant group was highly selected (see Appendix 1 and section 4.2.1).

Of 17 bone marrow transplant recipients, eight developed acute graft versus host disease; active CMV infection was present in 50% (4/8) of this group.

Of 33 liver transplant recipients, 11 lost the liver graft to chronic rejection and 22 patients comprised the control group; 73% and 46% of patients from these groups hosted active CMV infection respectively (see Chapter 4).

7.2.2 Clinical Specimens

7.2.2.1 Serum Samples

A total of 687 serum samples were collected; 381 from liver transplant recipients (median samples per patient 9.5 (2-35)) and 306 from bone marrow transplant recipients (median 16.5 (5-29)). All serum samples were tested in parallel for CMV DNA or HHV-6 DNA by PCR.
7.2.2.2 Whole Blood Samples

Whole blood samples (taken from a subset of the groups described above) were also available for study. These were collected from 23 liver and five bone marrow transplant recipients (see Appendixes 1 and 2 respectively).

Of 23 liver transplant recipients, seven lost the liver graft to chronic rejection and 16 patients comprised the control group; 83% and 69% of patients from these groups hosted active CMV infection respectively. Of 5 bone marrow transplant recipients, none developed acute graft versus host disease or active CMV infection.

A total of 218 whole blood samples were collected; 181 from liver transplant recipients (median samples per patient 6.5 (2-18)) and 37 from bone marrow transplant recipients (median 5 (4-15)). These samples were tested in parallel by PCR for human cellular DNA and HHV-6 DNA.

7.2.2.3 Tissue Samples

Liver tissue samples were also available for study; these samples were also tested for active CMV and results were described in Chapter 6.

Selection of tissue samples was described in Chapter 6 (see section 6.2.1 and Appendix 1). Briefly, liver graft sections from 29 liver transplant recipients were examined. Fifteen explanted grafts, from which sections were available, were selected for this study; 8 and 4 patients lost their graft to chronic rejection (CR) and hepatic artery thrombosis (HAT) respectively.

The control group comprised 3 patients that lost the graft to other diseases and 14 patients that retained their grafts. This highly selected control group was biased towards patients with evidence of active CMV infection and
comprised ten patients that developed active CMV infection (see Chapters 2 and 3) and seven patients that remained negative for CMV.

These samples were tested in parallel by PCR for human cellular DNA and HHV-6 DNA.

7.2.2.4 Collection and Storage of Samples

Collection and storage of sera was carried out as described in Chapter 2. Whole blood samples were taken into 5ml EDTA-blood tubes (Sarstedt) and stored at -20°C; these were taken in parallel with serum samples.

Formalin fixed, paraffin embedded tissue sample slides were kindly provided by Dr Nick Coleman. Methods used to fix and paraffin embed tissue were given in section 6.2.2.

7.2.3 DNA Extraction from Clinical Specimens

7.2.3.1 Serum and Whole Blood

DNA extraction from 200μl serum or 200μl whole blood was performed using the Qiamp DNA Extraction Kit (Qiagen; see Chapter 2). Extracted DNA from serum was amplified for CMV DNA (see Chapter 2) and HHV-6 DNA in parallel. Furthermore, each DNA extraction from whole blood was amplified in parallel for cellular DNA (β-globin gene; see section 7.2.4.2) and HHV-6 DNA.

7.2.3.2 Formalin Fixed, Paraffin Embedded Tissue

Formalin fixed, paraffin embedded tissue was cut into a 30μm section and placed in a 1.5 ml microfuge tube; blades were changed between each block to avoid contamination.
Sections were dewaxed by immersing in xylene (twice for 10 minutes each) before being washed in ethanol (twice for 10 minutes each) and air dried. Exposure of nucleic acids was achieved by digestion with 500μg/ml proteinase K (Boehringer Mannheim) in 50mM Tris (Sigma) for 12 hours at 37°C; proteinase K was then inactivated by heating the sample to 95°C for 10 minutes. Debris was pelleted from the sample by centrifuging at 13'000 rpm for 10 minutes. A total of 5μl of supernatant was used for each PCR reaction. Each DNA extraction from liver tissue was amplified in parallel for cellular DNA (β-globin gene; see section 7.2.4.2) and HHV-6 DNA.

7.2.4 Polymerase Chain Reaction (PCR)

7.2.4.1 PCR of HHV-6 DNA

Polymerase chain reaction (PCR) amplification of the major capsid gene of HHV-6 was carried out using sense (5'-TTAAGACTGTATGTAAACGCAT-3') and antisense (5'-TTTCACCACGCACGCATCGCTG-3') primers (kindly supplied by Dr B Thompson). Successful amplification yielded a 287 bp product. The major capsid gene is not homologous to cellular DNA or DNA from other herpes viruses (356).

DNA extracted from 50μl of serum or 3μl whole blood (made up to 50μl with water) underwent PCR. Sample DNA was added to 50μl of 'reaction mixture' that comprised reaction buffer™ (Promega), 1.5 mM MgCl₂ (Promega), 250 μM each deoxynucleotide triphosphate (Pharmacia), 1 μM each primer (Pharmacia) and 2.5 U Taq polymerase (Promega) and overlaid with one drop of mineral oil (Sigma). Reaction mixtures were
then subjected to a denaturing step (94°C for 4 minutes) before 35 thermal cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds).

7.2.4.2 PCR of CMV DNA
PCR amplification of a 293bp fragment of the immediate early gene of CMV was carried out as described in section 2.2.

7.2.4.3 PCR of Cellular DNA
Polymerase chain reaction (PCR) amplification of the human β-globin gene was carried out using primers which were kindly supplied by Dr B Thompson.

PCR was carried out as described for PCR of HHV-6 DNA (see section 7.2.4.1).

7.2.5 Southern blotting
After PCR for HHV-6 DNA, each sample was Southern blotted and probed using HHV-6 specific oligonucleotides (kindly supplied by Dr B Thompson).

Gel electrophoresis of 20μl PCR product was performed before Southern blotting onto nylon membrane as described earlier (see section 2.2.2.3). A cocktail of 2 oligonucleotides was end labelled with γ-P32 and used to probe membranes (see section 2.3.3.2). Two oligonucleotides were used in order to detect both the A and B variants of HHV-6; these sequences were

5'-CAGATGTCCAGACAGGCGAAG-3' (variant A) and
5'-CAGATGTGCTCCAGACAGGCGAGC-3' (variant B) respectively. These were kindly supplied by Dr B Thompson.
7.3 Results

7.3.1 Sensitivity of the PCR Test for HHV-6 DNA

This PCR test was highly sensitive; serial dilution of HHV-6 virions showed that 120 genome copies could be detected by PCR followed by gel electrophoresis alone and that 30 genome copies could be detected by PCR followed by Southern blotting and hybridisation of HHV-6 specific oligonucleotides (see figure 7.1).

Figure 7.1 Detection of 30 Copies of HHV-6 DNA After PCR and Southern Blotting
7.3.2 Serum Samples

7.3.2.2 PCR for CMV DNA (Control Reaction)
As shown in Chapter 3, 8.7% (60/687) serum samples were positive by PCR of CMV DNA; 48% (16/33) liver transplant and 24% (4/17) bone marrow transplant recipients were shown to develop active CMV infection post transplant.

7.3.2.1 PCR for HHV-6 DNA
None of 687 serum samples tested taken from 33 liver and 17 bone marrow transplant recipients were positive by PCR for HHV-6 DNA.

7.3.3 Whole Blood Samples

7.3.3.1 PCR for Cellular DNA (Control Reaction)
Initially, a random selection of 48 whole blood DNA samples underwent PCR for the human β-globin gene. DNA extracted from 10μl whole blood underwent PCR and β-globin DNA was amplified in 50% (24/48) samples.

It was suspected that PCR inhibitors were responsible for the lack of amplification of cellular DNA in half of the samples investigated. Therefore, PCR for the human β-globin gene was repeated using DNA from 3μl whole blood. Using this regime, all 218 samples were successfully amplified.

7.3.3.2 PCR for HHV-6 DNA
DNA from 3μl whole blood was amplified for HHV-6 DNA. None of 218 whole blood samples tested taken from 23 liver and 5 bone marrow transplant recipients were positive by PCR for HHV-6 DNA.
7.3.4 Liver Tissue Samples

7.3.4.1 PCR for Cellular DNA (Control Reaction)
PCR for the human β-globin gene was performed using 3μl extracted tissue DNA; using this regime all 29 samples were successfully amplified.

7.3.4.2 PCR for HHV-6 DNA
None of 14 liver biopsy samples or 15 samples from explanted livers that were lost to chronic rejection or hepatic artery thrombosis tested positive by HHV-6 PCR.

7.3.5 Antiviral Treatment or Prophylaxis for Liver Transplant Recipients
The distribution of 33 liver transplant recipients according to antiviral treatment or prophylaxis is given in table 7.1 and important points are described below.

Table 7.1: Distribution of Liver Transplant Recipients According to CMV Antibody Status and Treatment with Ganciclovir

<table>
<thead>
<tr>
<th>CMV Ab. status</th>
<th>Number</th>
<th>Active CMV</th>
<th>GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td>9</td>
<td>9 (100%)</td>
<td>7 (78%)</td>
</tr>
<tr>
<td>+/+</td>
<td>9</td>
<td>7 (78%)</td>
<td>4 (44%)</td>
</tr>
<tr>
<td>-/+</td>
<td>6</td>
<td>3 (50%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>-/-</td>
<td>4</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>unknown</td>
<td>5</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>19 (58%)</td>
<td>13 (39%)</td>
</tr>
</tbody>
</table>

Table 7.1 shows CMV antibody status (CMV Ab. status) of donor (D) and recipient (R) and active CMV infection status (Active CMV) for 33 liver transplant recipients. The number of patients of each group that were treated with ganciclovir is shown (GCV).
The results presented in table 7.1 show that 39% of all liver transplant recipients studied were treated with ganciclovir for active CMV infection. Furthermore, 27% (9/33) patients were CMV antibody negative recipients of grafts from CMV antibody positive donors; these patients were treated prophylactically with oral acyclovir. Therefore, a total of 15 patients (46%) received antivirals.
7.4 DISCUSSION

7.4.1 Conclusions

• Human herpesvirus-6 (HHV-6) is not an important pathogen after liver transplantation

• It is improbable that HHV-6 is involved in chronic rejection

7.4.2 Controls for HHV-6 PCR

Human herpes virus-6 (HHV-6) was absent in liver and bone marrow transplant recipients; this result was not consistent with other reports.

It is possible that active HHV-6 infection did occur but that viral load in samples was below the sensitivity of the PCR test used; however, this PCR test was shown to be highly sensitive (≤ 30 genome copies). Furthermore, reports of HHV-6 detection in peripheral blood mononuclear cells and lung tissue have been made after using PCR tests of similar sensitivity (339, 351).

A failure of DNA extraction from serum or in vitro degradation were excluded by the successful PCR for CMV DNA. Similarly, a failure of DNA extraction from whole blood or liver tissue samples or in vitro degradation was excluded by successful PCR for β-globin DNA.

Therefore, if 30 copies or more of HHV-6 DNA were present in serum, whole blood or liver tissue it is likely that this DNA would have been extracted efficiently and amplified.
It is also possible that sampling 'missed' episodes of active HHV-6 infection. However, many samples were tested and this 'sampling protocol' was adequate for detection of active CMV infection. Whole blood samples were included because HHV-6 has been shown to be highly 'cell-associated'; Yoshikawa et al (1991) (354) detected HHV-6 in bone marrow transplant recipients after culture of peripheral blood lymphocytes followed by immunohistochemistry but were unable to isolate HHV-6 from plasma.

Therefore, it is likely that these results genuinely reflected the absence of active HHV-6 infection in the patients screened.

7.4.3 Antivirals and Active HHV-6 Infection

Antiviral drug administration may have prevented active HHV-6 infection.

**Bone Marrow Transplantation**

All bone marrow transplant patients studied in this thesis received acyclovir prophylaxis from 3 days pretransplant. Furthermore, all patients at risk of CMV disease (ie. donor or recipient CMV antibody positive) received ganciclovir prophylaxis (low dose) from 30 days posttransplant until day 100 posttransplant. Symptomatic CMV infection was treated with full dose ganciclovir. In this study, 3 patients were CMV antibody negative and received marrow from a CMV antibody negative donor and therefore, did not receive ganciclovir.

Prophylactic ganciclovir administration was not performed during previous studies of HHV-6 reactivation after bone marrow transplantation in Wisconsin (351, 358). This group administered only acyclovir
prophylactically which has been shown to be less effective than ganciclovir for CMV (104, 105; see section 1.5.4) and HHV-6 (411, 412).

Ganciclovir prophylaxis and treatment for CMV may have prevented active HHV-6 infection in patients studied in this thesis; ganciclovir inhibits HHV-6 replication in vitro (411, 412). Active CMV infection was observed in these patients (see Chapter 3) but it is possible that CMV is less susceptible to ganciclovir than HHV-6.

Liver Transplantation
All CMV antibody mismatched liver transplant patients (recipient CMV antibody negative/ donor CMV antibody positive) received oral acyclovir prophylaxis and symptomatic CMV infection was treated with full dose ganciclovir. Nearly half of the patients studied received antivirals (see table 7.1).

It is possible that HHV-6 would assume active infection at a later stage in a hypothetical population of liver transplant recipients that received no antiviral treatment. It is also possible that patients with latent CMV pretransplant (ie. those more likely to be given ganciclovir) were more likely to also harbour HHV-6. It follows that antiviral treatment for CMV may be preemptive treatment for HHV-6.

One series of liver transplant recipients studied for active HHV-6 infection was reported by Sutherland et al (1991) (346). Here, ganciclovir was used as treatment for active CMV infection; details of any antiviral prophylaxis were not published. This paper provided indirect evidence that active HHV-6 infection was prevented by ganciclovir. Serological changes accompanying active HHV-6 infection in 18 patients occurred between 3-5
weeks post transplantation for 16/18 patients; serum was incubated with CMV before testing to remove antibodies cross-reactive between CMV and HHV-6. However, antibody rises occurred after 7 weeks post transplant for 2/18 patients; both patients with 'delayed active HHV-6 infection' had received ganciclovir treatment 3-4 weeks post transplant.

Furthermore, a recent study (425) supports the concept that prophylaxis or treatment for CMV may prevent active HHV-6 infection. Schmidt et al 1996 (425) found no evidence for HHV-6 reactivation after liver transplantation for 46 patients. All of these patients received prophylaxis for CMV; 'high risk' patients received five 10g doses of anti-CMV hyperimmune globulin in the first four weeks after transplantation, whereas 'normal risk' patients received two 10g doses on days 1 and 14 after transplantation. Details of any antiviral treatment administered were not given. It is possible that the anti-CMV hyperimmune globulin was reactive with HHV-6; cross-reactive antibodies between CMV and HHV-6 have been demonstrated previously by Sutherland et al (1991) (346).

7.4.4 HHV-6 Was Not Found in Liver Grafts Lost to Chronic Rejection

PCR testing of liver tissue from explanted grafts that had been lost to chronic rejection (and other liver graft samples) did not yield any positive results.

Therefore, it is unlikely that HHV-6 was associated with chronic rejection of liver grafts.
CHAPTER 8

FINAL DISCUSSION
8.1 Conclusions

Overall Conclusion
The hypothesis that cytomegalovirus (CMV) may initiate or enhance chronic rejection of liver grafts was upheld.

Chapters 2 and 3
Diagnosis of Active CMV Infection in Liver and Bone Marrow Transplant Recipients

- Detection of CMV DNA in serum by PCR was shown to be more sensitive than buffy coat DEAFF or culture.
- Detection of CMV DNA in serum by PCR allows prediction of active CMV infection.
- Semiquantitative PCR of CMV DNA in serum provides a threshold for prediction of active CMV infection.
- Post transplant monitoring of patients for CMV DNA in serum by qualitative and/or semiquantitative PCR facilitates pre-emptive antiviral treatment.

Chapter 4 Risk Factors for Chronic Rejection of Liver Grafts

Active CMV Infection
- Prolonged active CMV infection after liver transplantation (30 days or more), detected by serum and/or urine PCR was a risk factor for chronic rejection.
- The incidence of urine PCR positivity was a risk factor for chronic rejection.

CMV and Tumour Necrosis Factor (TNF)
- Recipients that possess the TNF-2 promoter allele, which is associated with enhanced expression of TNF, are at increased risk of developing chronic rejection.
- Furthermore, active CMV infection may synergise with the TNF-2 promoter allele as a risk factor for chronic rejection.

CMV and Human Leukocyte Antigens (HLA)
• Matching and mismatching of HLA alleles per se may not be an important risk factor for chronic rejection.

• However, HLA class I matching may be an important factor for clearance/control of active CMV which in turn may be a risk factor for chronic rejection.

Other Risk Factors
• Two or more episodes of acute rejection are a risk factor for chronic rejection.

• A pre transplant diagnosis of primary biliary cirrhosis (PBC) is a risk factor for chronic rejection.

Chapter 5 Humoral Immunity and Chronic Rejection of Liver Grafts

Antibodies to Bile Duct and Hepatic Artery
• Pre transplant IgG antibodies to 160 and 85 kD proteins of chronically rejected hepatic artery may be associated with an increased risk of chronic rejection.

• Pre transplant IgA antibodies to 94 and 39 kD proteins of chronically rejected, CMV infected bile duct are associated with an increased risk of chronic rejection.

Relationship with CMV
• An IgA antibody to a 44 kD protein of chronically rejected, CMV infected bile duct which developed post transplant was associated with development of active CMV infection but was not associated with chronic rejection.

• Polyclonal antibodies raised against whole CMV were not cross-reactive with hepatic artery or bile duct antigens.

Relationship with Primary Biliary Cirrhosis (PBC) and Primary Sclerosing Cholangitis (PSC)
• IgA antibodies to 44 and 34 kD proteins of chronically rejected, CMV infected bile duct were associated with a pre transplant diagnosis of PBC and PSC respectively but were not associated with chronic rejection.
Tropism of Active CMV in the Liver Graft

- Replicating CMV was found in vascular endothelial cells, epithelial cells, hepatocytes and mononuclear cells in liver grafts.

CMV Antibody Status and Active CMV Infection of the Liver Graft

- CMV antibody negative recipients that received grafts from CMV antibody positive donors and subsequently developed active CMV infection (detected by viral surveillance of serum and/or buffy coat and/or urine) were at high risk of active CMV infection of the liver graft.

- However, active CMV infection of the liver graft occurred without detection of active CMV infection by viral surveillance of serum and/or buffy coat and/or urine.

- Furthermore, active CMV infection of the liver graft occurred in CMV antibody negative recipients that received grafts from CMV antibody negative donors.

Active CMV Infection of the Liver Graft and HLA Status

- Mismatching for the HLA B allele may be associated with an increased risk of active CMV infection of the liver graft as predicted in Chapter 4; however, this result was not significant and a firm conclusion cannot be drawn.

Active CMV Infection of the Liver Graft and Chronic Rejection

- Active CMV infection of bile duct epithelial cells was associated with CR. However, this result did not reach significance.

- In sections taken from liver grafts that were explanted after chronic rejection, active CMV infection of bile duct epithelial cells or hepatic artery endothelial cells was only demonstrated in portal tracts of normal appearance and was not demonstrated in adjacent portal tracts that were obviously damaged; it is possible that active CMV infection of these sites is an important event during the early stages of development of chronic rejection and that cells harbouring active CMV become destroyed at a later stage.

Chapter 7 Human herpesvirus-6 (HHV-6) and Liver Transplantation

- HHV-6 is not an important pathogen after liver transplantation
8.2 Diagnosis of Active CMV Infection

Liver and bone marrow transplant recipients at Addenbrooke's NHS Trust are currently monitored for active CMV infection by DEAFF and culture testing of buffy coat and urine samples and by serological change. In addition, immunostaining of liver biopsy specimens is carried out after clinical suspicion of CMV hepatitis.

8.2.1 Recommendations

However, on the basis of the work presented in Chapters 2 and 3 the following tests are suggested:

(1) Qualitative and semi-quantitative PCR of CMV DNA in serum performed weekly after transplantation

Qualitative and semiquantitative PCR were shown to be more sensitive than DEAFF, culture and CMV serology and to provide sensitive and specific prediction of CMV disease in liver and bone marrow transplant recipients.

Furthermore, PCR is suitable for routine use because it is rapid, reliable, and relatively cheap.

The PCR tests used in this thesis were completed after 12 hours for qualitative and 16 hours for semiquantitative PCR whereas DEAFF and culture were completed after 48 hours and 2-21 days respectively. Over 100 runs were performed during the course of this thesis and failure or contamination problems were only experienced four times; these
problems were easily rectified by changing stock solutions. Finally, the cost per sample for PCR was approximately half that for culture or DEAFF.

The combination of rapid testing and high sensitivity may enable preemptive antiviral treatment.

(2) In situ Hybridisation (ISH) and Immunostaining of Liver Biopsy Specimens for CMV

It is recommended that this is performed for all liver biopsies taken after transplantation. ISH was shown to be more sensitive than immunostaining (see Chapter 6) and may allow preemptive antiviral treatment before clinical signs of CMV hepatitis are apparent; these tests should be compared in a large prospective study.

8.2.2 Recent Developments

Recent evidence suggests that semi-quantitative PCR of CMV DNA may facilitate preemptive antiviral therapy and therefore is in accord with the conclusions drawn from this thesis. Ljungman et al (1996) (413) performed semi-quantitative PCR of CMV DNA in peripheral blood lymphocytes taken from 58 bone marrow transplant recipients and administered ganciclovir after two consecutive positive tests. Initiation of ganciclovir treatment was significantly earlier (mean equaled 10 days) when compared to 58 matched, historical controls and was shown to successfully clear active CMV for 28/36 treated episodes.

Transplant centres that adopt this technology should carefully validate their test. This is emphasised in a recent report of a quality control study set up by the European Group for Blood and Marrow Transplantation
Forty eight peripheral blood samples were taken from bone marrow transplant recipients and tested in parallel by three centres; 38/48 samples generated identical results (35 negative and three positive). However, of 12 samples tested positive by one or more centres, only three were in agreement.

The Overall Hypothesis
Setting up sensitive PCR and in situ hybridisation tests for active CMV infection in serum and liver tissue was an important prerequisite for testing the overall hypothesis that CMV may be involved in chronic rejection.
8.3 Cytomegalovirus and Chronic Rejection of Liver Grafts

8.3.1 Models

The conclusions of this thesis and other work allow several models to be set up to explain the interaction between CMV and chronic rejection of liver grafts. A link between mismatched CMV serology and chronic rejection (210) suggests that CMV is causal but proposed links with active CMV infection can be interpreted ambiguously which is reflected here. It is important to realise that the four proposed models are not mutually exclusive and that a 'vicious circle' may occur where CMV enhances the process of chronic rejection which in turn enhances CMV replication.

Model 1 CMV Initiates or Augments Cellular Rejection

This model is shown diagramatically and possible interactions with CMV (numbered) are described below.
(1) Matched HLA DR presents CMV peptides to CMV-specific T-lymphocytes (224; see section 1.9.1.1)

(2) Presenting cells (endothelial or epithelial cells) harbour replicating CMV; this was shown in Chapter 6 and permissivity has been demonstrated in vitro (249; 251, 271, 217; see section 1.8.2.3). Active CMV infection activates CD4+ T cells which in turn leads to upregulation of HLA DR expression (431; see section 1.9.2) thus enhancing presentation of CMV or foreign peptides.

(3) Active CMV infection of endothelial or epithelial cells upregulates adhesion molecule expression (435, 436; see section 1.9.4) and thus augments presentation.

(4) Other reports have suggested HLA restricted presentation of foreign HLA molecules (224, 243) or Y-chromosome specific antigens (210; see section 1.9.1).

(5) Active CMV infection enhances interleukin (IL) production (264, 266, 267; see section 1.9.4) thus enhancing T-lymphocyte activation.

(6) Active CMV infection also enhances interferon-a (IFN-a); Arnold et al (1993) (265) found that IFN-a expression in bile duct epithelial cells was enhanced during episodes of active CMV infection.

(7) Tumour necrosis factor-a (TNF-a) is also an inflammatory molecule that activates T-lymphocytes. Results presented in Chapter 4 of this thesis show that recipients that have the TNF-2 allele (associated with upregulated TNF expression) are more likely to develop chronic rejection and that active CMV infection synergises with this risk factor. This work is supported by reports that high serum TNF-a levels are associated with chronic rejection of liver grafts (397) and that active CMV infection further upregulates TNF-a production (402).

(8) and (9) Active CMV infection may augment production of interleukins and TNF (see (5) and (7)); this further activates T-lymphocytes directed to CMV peptides (8) and may also activate local alloreactive T-lymphocytes (9).

This model applies to cellular responses to bile duct epithelium and hepatic artery endothelium. The latter layer, of chronically rejected liver grafts, contains endothelial cells and infiltrating macrophages i.e. foam cells (191; see section 1.8.1.2); both of these cells may present peptides via MHC class II (242).
Finally, the hypothesis put forward by Arnold et al (1992) (284) that CMV infection of hepatocytes may enhance the activity of alloreactive T-lymphocytes directed to bile duct epithelial cells (by local upregulation of cytokines and adhesion molecules) is applicable to this model.

Model 2  Cellular Rejection Initiates or Augments Opportunistic Active CMV Infection

This model is shown diagramatically and possible interactions with CMV (numbered) are described below.

(1) The endothelium of chronically rejected liver grafts comprises endothelial cells and infiltrating foam cells (macrophages). Macrophages have been shown to harbour active CMV; these cells therefore import replicating CMV to areas of chronic rejection (397; see section 4.4.4).
Chapter 6 shows that endothelial cells may harbour replicating CMV. It is possible that this is a reactivation of latent virus (murine CMV is latent in liver endothelial cells (44)) initiated by a repair process; there is evidence to suggest that cell differentiation is crucial for CMV to switch from latent to lytic infection (48).

(2) T-lymphocytes have also been shown to be a site for CMV replication (272) and, in this model, also import CMV to sites of chronic rejection.

(3) Furthermore, CMV virions produced in lymphocytes may infect graft cells and vice versa. Graft cells and lymphocytes are intimate and MHC class I molecules have been suggested to be receptors for CMV (62).

(4) Activated T-lymphocytes produce TNF-α which has been shown to upregulate CMV activity in vitro (398, 399; also see section 4.4.4).

(5) In the context of this model, clonal propagation of T-lymphocytes is seen as an amplification of viable sites for CMV replication.

(6) Finally, treatment of rejection with steroids may facilitate CMV replication; hydrocortisone has been shown to differentiate peripheral blood monocytes into fully permissive macrophages (415).

Model 3  

CMV Infection may Initiate or Augment a Process Similar to Atherosclerosis

This model is put forward because of the following observations:

1. Ischaemic injury has been documented during the early stages of chronic rejection (191)

2. Hepatic arteries that are affected are histologically similar to atherosclerotic arteries

3. Chronic rejected liver grafts do not often contain a large infiltrate of mononuclear cells
4. A herpesvirus has been shown to mediate atherosclerosis in an avian model (392).

Melnick et al (1995) (388) proposed a model for accelerated atherosclerosis after cardiac transplantation (see section 6.4.6). Here, damage to coronary artery endothelial cells leads to exposure and damage of smooth cells. A repair process induces proliferation of smooth muscle cells which leads to thickening of the intima and therefore occlusion of the lumen. The authors suggest that the initial injury to endothelial cells may be due to humoral responses or active CMV which has been shown to infect the coronary artery endothelium (385; see section 6.4.6).

This model is now proposed for damage to hepatic arteries observed during chronic rejection of liver grafts; further evidence for this model is summarised below.

(1) Chapter 6 of this thesis demonstrates CMV infection of endothelial cells. Endothelial cells are fully permissive to CMV (249, 251, 271) and therefore infection is likely to be associated with damage.

(2) Chapter 5 of this thesis describes antibodies to hepatic artery proteins of 85 and 160 kD that were associated with chronic rejection. It is possible that these antibodies are directed to endothelial cells and therefore initiate an atherosclerosis-like process.

(3) There is also some evidence that CMV can transform smooth muscle cells (281); abnormal proliferation of these cells could further thicken the intima.
Model 4  A Process Similar to Atherosclerosis may Facilitate Active CMV Infection

It is also possible that the link between active CMV infection and chronic rejection is due to CMV opportunism. In the context of this model, proliferation of smooth muscle cells observed during atherosclerosis is an amplification of sites permissive to CMV replication.

8.3.2 Recent Developments

A recent report provides further evidence for interaction between CMV and cellular immune responses important in chronic rejection of solid allografts (model 1). Steinhoff G et al (1996) (416) studied a rat model of allogeneic lung transplantation and found that infection of recipients with rat cytomegalovirus was associated with significant elevation of the adhesion molecule ICAM-1 on pulmonary artery endothelium and infiltration of CD11+ lymphocytes (CD4+).

Another recent report confirms the rationale used to propose that some cases of chronic rejection of liver grafts are due to a process similar to atherosclerosis. Radio S et al (1996) (417) has recently shown that coronary arteries involved with accelerated atherosclerosis after cardiac transplantation are similar histologically to medium sized and large arteries involved with chronic rejection of liver, pancreas and renal grafts.
APPENDIX 1

Clinical Details of Liver Transplant Recipients

Appendix 1 gives brief clinical information of the liver transplant patients in this study. All CMV antibody mismatched recipients (ie. donor positive /recipient negative for CMV antibodies) received prophylactic acyclovir. Full dose ganciclovir treatment was given when symptomatic CMV disease was suspected.

Patients are ordered according to chronic rejector status (CR), ‘non-CR’ retransplantation status (Redo), active CMV status (by serum and/or urine PCR, buffy coat and/or urine DEAFF or culture), PBC status and then PSC status.

Numerals relate to the chapters in which a particular patient was studied. VII (a) marks patients studied by serum PCR, VII (b) marks patients studied by whole blood PCR and VII (c) marks patients studied by liver tissue PCR in chapter VII.

CMV antibody status of donor/recipient is denoted D/R. The timing of clinical findings is given as days after transplantation. Liver function tests is abbreviated to LFT's.

Patient 1

II, III, IV, V, VI, VII (a), VII (b), VII (c)

Transplant 1

This woman was 60 years old when she underwent her first liver transplant for primary biliary cirrhosis. CMV antibody status was D7/R+; a donor serum sample was not available. Active CMV infection was observed on day 45 by urine PCR without the appearance of symptoms. LFT's were raised on day 114 and ganciclovir treatment commenced on day 118; clearance
was not proven. The graft was acutely rejected 3 times (days 7, 28, 110) and this patient died of chronic rejection on day 161.

Patient 2 II, III, IV, V, VI, VII (a), VII (b), VII (c)
Transplant 1
This woman was 41 years old when she underwent her first liver transplant for primary biliary cirrhosis. CMV antibody status was D+/R-; active CMV infection was observed on day 26 by urine and serum PCR and lasted 38 days. Pyrexia and abnormal LFT's were observed on day 38 and ganciclovir treatment commenced on day 40 and cleared by day 67. The graft was not acutely rejected but was lost to chronic rejection on day 132.

Transplant 2
CMV antibody status was D?/R+; a donor serum sample was not available. Active CMV infection was observed on day 27 by urine PCR and lasted 28 days. Symptoms were not observed and antiviral treatment was not administered. The graft was neither acutely nor chronically rejected.

Patient 3 II, III, IV, V, VI, VII (a), VII (b), VII (c)
Transplant 1
This man was 60 years old when he underwent his first liver transplant for primary sclerosing cholangitis. CMV antibody status was D+/R-. Active CMV infection was observed intermittently from day 50 to 167 by serum and urine PCR. Furthermore, CMV hepatitis was diagnosed on day 40 and ganciclovir treatment commenced on day 50; CMV appeared to clear from the graft. The graft was not acutely rejected but was lost to chronic rejection on day 170.

Transplant 2
CMV antibody status was D+/R+. Active CMV infection was on day 0 by serum PCR and lasted 14 days. The graft was lost on day 15.

Transplant 3
CMV antibody status was D?/R+; a donor serum sample was not available. Active CMV infection was observed on day 25 by serum and urine PCR but no symptoms were observed and antivirals were not administered. The graft was neither acutely nor chronically rejected. This patient died from multi organ failure.
Patient 4 II, III, IV, V, VII (a), VII (b)
Transplant 1
This man was 47 years old when he underwent his first liver transplant for hepatitis C cirrhosis. CMV antibody status was D- /R+. Active CMV infection did not occur. The graft was acutely rejected three times (days 2, 12 and 362) and was lost on day 400 to chronic rejection.

Transplant 2
CMV antibody status was D?/R+; donor serum was not available. Active CMV infection was not observed after testing blood or urine but CMV was observed in colon tissue on day 32. Furthermore, pyrexia, pneumonia and abnormal LFT's were observed on day 30; ganciclovir was administered on day 30. The graft was not acutely or chronically rejected. This patient died from multi organ failure.

Patient 5 II, III, IV, V, VI, VII (a), VII (c)
Transplant 1
This man was 33 years old when he underwent his first liver transplant for haemachromatosis and hepatitis C cirrhosis. CMV antibody status was D+/R+. Active CMV infection was observed on day 62 (by urine PCR) and did not clear; CMV related symptoms were not observed and antivirals were not administered. The graft was acutely rejected once (day 2) and lost to chronic rejection 110 days posttransplant.

Transplant 2
The patient died in theatre.

Patient 6 II, III, IV, V, VII (a)
Transplant 1
This woman was 39 years old when she underwent her first liver transplant for acute hepatic failure. CMV antibody status was D+/R+; further virological details are unknown. The graft was acutely rejected twice (days 3 and 33) and lost to chronic rejection 143 days posttransplant.

Transplant 2
CMV antibody status was D- /R+; active CMV infection was observed on day 26 (by serum and urine PCR) and lasted for 33 days; no symptoms were observed and antivirals were not administered. The graft was not acutely rejected but was lost to chronic rejection 143 days posttransplant.
Transplant 3
CMV antibody status was D+/R+; active CMV infection was observed on day 0 (by urine PCR) and lasted for 18 days; no symptoms were observed and antivirals were not administered. The graft was acutely rejected once (day 8). This patient died of a stroke.

Patient 7  II, III, IV, VII (a), VII (b)

Transplant 1
This man was 58 years old when he underwent his first liver transplant for hepatitis C cirrhosis. CMV antibody status was D-/R+. Active CMV infection was not observed by laboratory testing but idiopathic abnormal LFT's were observed immediately after transplantation and worsened from day 38; antiviral treatment was not administered. The graft was not acutely rejected but was lost on day 53 to chronic rejection.

Transplant 2
CMV antibody status was D-/R+. Active CMV infection was observed on day 4 by serum PCR and lasted 14 days. Symptoms (pyrexia) were observed on day 24 and ganciclovir treatment was initiated on day 25. Acute rejection occurred on day 26. The graft was lost on day 164 due to hepatic artery thrombosis and multiliver abscesses.

Transplant 3
CMV antibody status was D-/R+. The posttransplant course was uncomplicated and this patient is well at 32 months post transplant.

Patient 8 II, III, IV, VI, VII (a), VII (c)

Transplant 1
This man was 45 years old when he underwent his first liver transplant for cirrhosis of unknown causes. CMV antibody status was D+/R+. Active CMV infection was not observed. The graft was not acutely rejected but was lost on day 19 to hepatic artery thrombosis.

Transplant 2
CMV antibody status was D+/R+. Data for active CMV infection status was not available. The graft was not acutely rejected (day 3) and but was lost to hepatitis C related cirrhosis on day 705.

Transplant 3
CMV antibody status was D?/R+; donor serum was not available. Active CMV infection was observed intermittently from day 59 to day 110 by serum PCR. CMV hepatitis was diagnosed on day 45 and ganciclovir treatment commenced on day 50. The graft was acutely rejected once (day 45).
Patient 9 II, III, IV, VI, VII (a), VII (c)
Transplant 1
This woman was 59 years old when she underwent her first liver transplant for cirrhosis of unknown causes. CMV antibody status was D-/R+; further virological details are unknown. The graft was acutely rejected once (day 30) and was lost to chronic rejection on day 229.

Transplant 2
CMV antibody status was D?-/R+-; a donor serum sample was not available. Active CMV infection was not observed by laboratory testing but idiopathic cholestatic LFT's were raised on day 67; antivirals were not administered. The graft was neither acutely nor chronically rejected.

Patient 10 V, VI, VII (c)
Transplant 1
This woman was 62 years old when she underwent liver transplantation for primary biliary cirrhosis. CMV antibody status was D7/-/R+; a donor serum sample was not available. Symptomatic CMV infection was observed clinically and confirmed by antibody status; CMV hepatitis was observed on liver biopsy at day 54. This patient was retransplanted for chronic rejection on day 135.

Patient 11 II, III, IV, V, VI, VII (a), VII (c)
Transplant 1
This woman was 22 years old when she underwent liver transplantation for subacute hepatitis. CMV antibody status was D-/R-; active CMV infection was not observed. However, idiopathic hepatitis and abnormal LFT's were observed on day 98. The graft was lost on day 129; recurrent disease was thought to be responsible.

Transplant 2
CMV antibody status was D-/-/R-; active CMV infection was not observed. The graft was acutely rejected on day 8 and lost on day 14.

Transplant 3
CMV antibody status was D-/-/R-; active CMV infection was not observed. However, idiopathic abnormal LFT's were observed on day 34. The graft was acutely rejected on day 4 but this patient is well at 15 months post transplant.

Patient 12 II, III, IV, VI, VII (a), VII (b), VII (c)
Transplant 1
This woman was 53 years old when she underwent her first liver transplant for hepatitis C cirrhosis. CMV antibody status was D+/R+; active CMV infection was not observed by serum or urine PCR or DEAFF. However, idiopathic symptoms prevailed (LFT's were raised on day 16 and pyrexia was observed on day 30) and ganciclovir was administered on day 32. The graft was acutely rejected once (day 7) and lost to chronic rejection on day 289.

Transplant 2
CMV antibody status was D+/R+. Active CMV infection was observed on day 8; no symptoms were observed and antivirals were not administered. The graft was lost on day 20 to hepatic artery thrombosis.

Transplant 3
CMV antibody status was D+? /R+; a donor serum sample was not available. Active CMV infection was not followed. This patient died on day 3.

Patient 13 II, III, IV, V, VI, VII (a), VII (b), VII (c)
Transplant 1
This man was 54 years old when he underwent his first liver transplant for primary biliary cirrhosis. CMV antibody status was D+? /R+; donor serum was not available. Active CMV infection was not observed. The graft was not acutely rejected but was lost to chronic rejection on day 132.

Transplant 2
CMV antibody status was unknown. Active CMV infection was not observed. The graft was acutely rejected once (day 41). This patient remains well at 3 years post transplant.

Patient 14 II, III, IV, V, VII (a),
Transplant 1
This man was 31 years old when he underwent his first liver transplant for hepatitis B cirrhosis. CMV antibody status was D- /R+. Active CMV infection was not observed. The graft was acutely rejected three times (days 2, 7 and 62) and this patient died of chronic rejection.

Patient 15 II, III, IV, VI, VII (a), VII (c)
Transplant 1
This man was 39 years old when he underwent his first liver transplant for Budd Chiari syndrome. CMV antibody status was D-/R-. Active CMV infection was not observed. The graft was not acutely rejected but was lost to unspecified biliary tract disease.
Transplant 2
CMV antibody status was D+ /R-. Active CMV infection was not observed. The graft was acutely rejected (day 3) and was lost to hepatic artery thrombosis on day 12.

Transplant 3
CMV antibody status was D+ /R-. Active CMV infection was observed on day 37 (by urine PCR) and cleared after 10 days. Symptoms were not observed and antivirals were not administered. The graft was acutely rejected once (day 7).

Patient 16

Transplant 1
This man was 66 years old when he underwent his first liver transplant for PBC. CMV antibody status was D+ /R+. The graft was lost to hepatic artery thrombosis on day 2.

Transplant 2
CMV antibody status was D+ /R+. Active CMV infection was observed by CMV antibody surveillance on day 111; symptoms were not observed and antivirals were not administered. The graft was acutely rejected on day 69 but retained.

Patient 17

Transplant 1
This man was 45 years old when he underwent his first liver transplant for hepatitis C cirrhosis. CMV antibody status was D+ /R+. Active CMV infection was not observed. The graft was acutely rejected on day 7 and lost on day 12.

Transplant 2
CMV antibody status was D+ /R+. Active CMV infection occurred on days 24 and 56 diagnosed by urine and serum PCR respectively and lasting for 11 and 20 days respectively. Abnormal LFT's were observed on days 9 and 25. Acute or chronic rejection did not occur.

Patient 18

Transplant 1
This woman was 49 years old when she underwent liver transplantation for autoimmune cirrhosis. CMV antibody status was D- /R+; active CMV infection was not observed. The graft was lost on day 11 to non-thrombotic graft infarction.

Transplant 2
CMV antibody status was D+/ R+. Active CMV infection occurred; pyrexia was observed on day 29 and ganciclovir treatment was initiated on day 29. Serological changes confirmed CMV on day 32. Acute or chronic rejection did not occur.

Patient 19  II, III, IV, VI, VII (a), VII (c)
Transplant 1
This man was 55 years old when he underwent his first liver transplant for alcoholic cirrhosis. CMV antibody status was D-/R-. Active CMV infection was not observed. The graft was lost on day 9 to hepatic artery thrombosis.

Transplant 2
CMV antibody status was unknown. Active CMV infection was not observed. Again, the graft was lost to hepatic artery thrombosis on day 33.

Transplant 3
CMV antibody status was unknown. This patient died on day 3 from pneumonia.

Patient 20  II, III, IV, VI, VII (a), VII (c)
Transplant 1
This woman was 61 years old when she underwent liver transplantation for subacute hepatitis. CMV antibody status was D? /R+; donor serum was not available. Active CMV infection was not observed. Symptomatic infection did not occur. The graft was lost to acute rejection on day 14.

Transplant 2
CMV antibody status was D? /R+; donor serum was not available. Active CMV infection was not observed. Symptomatic infection did not occur. The graft was acutely rejected on day 149.

Patient 21  II, III, IV, V, VI, VII (a), VII (c)
Transplant 1
This woman was 23 years old when she underwent liver transplantation for cryptogenic biliary cirrhosis. CMV antibody status was D+ /R+. Active CMV infection was observed on day 37 by serum and urine PCR; overall duration was 23 days. Symptoms (abnormal LFT's and pyrexia) were observed on day 38. The graft was acutely rejected on day 8.

Patient 22  V
Transplant 1

321
This woman was 47 years old when she underwent liver transplantation for primary biliary cirrhosis. CMV antibody status was D? /R+; a donor serum sample was not available. Active CMV infection of urine was observed on day 40 by culture and did not clear. Symptoms (abnormal LFT’s and pyrexia) were observed on day 36. Acute or chronic rejection did not occur.

Patient 23  V  
Transplant 1  
This woman was 51 years old when she underwent liver transplantation for primary biliary cirrhosis. CMV antibody status was D? /R+; donor serum was not available. Active CMV infection was observed on day 43 by antibody status. Symptomatic infection did not occur and antivirals were not administered. Acute or chronic rejection did not occur.

Patient 24  V  
Transplant 1  
This woman was 43 years old when she underwent liver transplantation for primary biliary cirrhosis. CMV antibody status was D+ /R-; active CMV infection was not observed. Acute rejection occurred on day 7.

Patient 25  V  
Transplant 1  
This man was 54 years old when he underwent his first liver transplant for alcoholic cirrhosis. CMV antibody status was D? /R?; serum was not available. Active CMV infection was observed as DEAFF positive urine (d68) without symptoms and antivirals were not administered. Acute or chronic rejection did not occur.

Patient 26  II, III, IV, V, VI, VII (a), VII (b), VII (c)  
Transplant 1  
This woman was 63 years old when she underwent liver transplantation for hepatic carcinoid. CMV antibody status was D+ /R-. Active CMV infection was observed on day 39 by urine PCR and day 44 by serum PCR; overall duration was 32 days. Symptoms were observed; abnormal LFT’s (day 15) and pyrexia (day 20). The graft was neither acutely nor chronically rejected. This patient died of CMV pneumonitis despite ganciclovir treatment which commenced on day 42.

Patient 27  II, III, IV, V, VI, VII (a), VII (b), VII (c)  
Transplant 1  
This man was 55 years old when he underwent liver transplantation for alcoholic cirrhosis. CMV antibody status was D+ /R-. Active CMV infection was observed on day 39 by serum
PCR and day 74 by urine PCR. Overall duration was 70 days. Cholestatic LFT's were observed on day 113 and 1 episode of acute rejection was observed on day 114.

**Patient 28**  
**II, III, IV, V, VII (a)**  
**Transplant 1**  
This man was 52 years old when he underwent liver transplantation for cirrhosis of unknown causes. CMV antibody status was D-/R+. Active CMV infection was observed on day 43 by urine PCR and lasted 7 days. Pyrexia and abnormal LFT's were observed on day 25. Acute rejection occurred on day 7.

**Patient 29**  
**II, III, IV, V, VI, VII (a), VII (c)**  
**Transplant 1**  
This man was 30 years old when he underwent liver transplantation for cirrhosis of unknown causes. CMV antibody status was D+/R-. Active CMV infection was observed on day 46 by serum and urine PCR and lasted for over 100 days despite administration of ganciclovir on day 46. Abnormal LFT's were observed on day 26 and CMV hepatitis was diagnosed on day 46. Acute or chronic rejection did not occur.

**Patient 30**  
**V, VI, VII (c)**  
**Transplant 1**  
This woman was 33 years old when she underwent liver transplantation for glycogen storage disease. CMV antibody status was D+/R-; active CMV infection was observed and accompanied by abnormal LFT's and pyrexia. The graft was neither acutely nor chronically rejected.

**Patient 31**  
**V, VI, VII (c)**  
**Transplant 1**  
This woman was 43 years old when she underwent liver transplantation for primary biliary cirrhosis. CMV antibody status was D?/R-; donor serum was not available. CMV-like symptoms (abnormal LFT's and pyrexia) were observed but CMV was not confirmed by laboratory testing. The graft was neither acutely nor chronically rejected.

**Patient 32**  
**V, VI, VII (c)**  
**Transplant 1**  
This woman was 56 years old when she underwent liver transplantation. CMV antibody status was D?/R?; serum was not available. Active CMV infection was observed by DEAFF positive urine on day 67. This patient developed CMV retinitis, was transiently blind and is now partially sighted after administration of ganciclovir. The graft was neither acutely nor chronically rejected.
Patient 33  V, VI, VII (c)
This man was 59 years old when he underwent liver transplantation for hepatitis C cirrhosis. CMV antibody status was D+ /R-. Active CMV infection was observed on day 76 by antibody status. The graft was lost to chronic rejection at 180 days post-transplant.

Patient 34  II, III, IV, V, VI, VII (a), VII (c)
Transplant 1
This woman was 32 years old when she underwent liver transplantation for Budd Chiari syndrome. CMV antibody status was D+ /R-. Active CMV infection was observed on day 60 by serum PCR. Symptomatic infection did not occur and antivirals were not administered. The graft was neither acutely nor chronically rejected.

Patient 35  II, III, IV, V, VI, VII (a), VII (b), VII (c)
Transplant 1
This man was 31 years old when he underwent liver transplantation for alcoholic cirrhosis. CMV antibody status was D+ /R-. Active CMV infection was observed on days 36 and 82 by serum PCR; lasting for 10 and 14 days respectively. CMV hepatitis was diagnosed on day 46 and pyrexia was observed on days 40 and 90. Acute rejection occurred on day 22.

Patient 36  II, III, IV, VI, VII (a), VII (c)
Transplant 1
This man was 34 years old when he underwent liver transplantation for cirrhosis of unknown causes. CMV antibody status was D+ /R-. Active CMV infection was observed on day 46 by urine PCR and lasted 8 days. Furthermore, unexplained hepatitis was observed on day 70. Neither acute nor chronic rejection were observed.

Patient 37  II, III, IV, VII (a)
Transplant 1
This woman was 51 years old when she underwent liver transplantation for fulminant hepatitis. CMV antibody status was D? /R+; donor serum was not available. Active CMV infection was observed on day 47 by urine PCR. Symptomatic infection did not occur and antivirals were not administered. The graft was acutely rejected on day 5.

Patient 38  V
Transplant 1
This woman was 54 years old when she underwent liver transplantation for PBC. CMV antibody status was D? /R+; donor serum was not available. Active CMV infection was not observed. The graft was neither acutely nor chronically rejected.
Patient 39

Transplant 1

This man was 33 years old when he underwent liver transplantation for PSC. CMV antibody status was D? /R+. Donor serum was not available. Active CMV infection was not observed and neither acute nor chronic rejection were observed.

Patient 40

Transplant 1

This woman was 44 years old when she underwent liver transplantation for primary sclerosing cholangitis. CMV antibody status was D-/R+. Active CMV infection was observed on day 11 but symptoms were not observed and antivirals were not administered. The graft was acutely rejected on day 6.

Patient 41

Transplant 1

This man was 49 years old when he underwent liver transplantation for PSC. CMV antibody status was D-/R-. Active CMV infection and acute or chronic rejection were not observed.

Patient 42

Transplant 1

This woman was 57 years old when she underwent liver transplantation for PSC. CMV antibody status was D-/R-. Active CMV infection was not observed. The graft was acutely rejected on days 1 and 26.

Patient 43

Transplant 1

This woman was 62 years old when she underwent liver transplantation for acute hepatic failure. CMV antibody status was D+/R+. Active CMV infection was not observed. The graft was acutely rejected on day 9.

Patient 44

Transplant 1

This woman was 17 years old when she underwent liver transplantation for adrenal and liver malignancies. CMV antibody status was D-/R-. Active CMV infection was not observed. The graft was acutely rejected on day 7. This patient died of primary disease at 12 months after transplantation.

Patient 45

Transplant 1
This man was 51 years old when he underwent liver transplantation for alcoholic cirrhosis. CMV antibody status was D- /R+. Active CMV infection was not observed. The graft was acutely rejected on day 12.

Patient 46  II, III, IV, V, VI, VII (a), VII (b), VII (c)
Transplant 1

This man was 55 years old when he underwent liver transplantation for alcoholic cirrhosis. CMV antibody status was D+/R+. The course posttransplantation was uneventful.

Patient 47  II, III, IV, VI, VII (a), VII (c)

This man was 52 years old when he underwent liver transplantation for hepatitis C cirrhosis. CMV antibody status was D-/R-. Active CMV infection was not observed. Acute rejection was observed on day 9.

Patient 48  II, III, IV, VI, VII (a), VII (b), VII (c)
Transplant 1

This man was also 52 years old when he underwent liver transplantation for hepatitis C cirrhosis. CMV antibody status was D-/R+. Active CMV infection was not observed. Acute or chronic rejection was not observed.
APPENDIX 2

Clinical Details of Bone Marrow Transplant Recipients

Appendix 2 gives brief clinical information of the bone marrow transplant patients in this study. All patients were given acyclovir prophylaxis prior to transplantation and all at risk of CMV disease (ie. all except donor negative /recipient negative CMV antibody status) received prophylactic, low dose ganciclovir from day 30. Full dose ganciclovir treatment was given when symptomatic CMV disease was suspected or laboratory results were positive.

Numerals relate to the chapter(s) in which a particular patient was studied. VII (a) marks patients studied by serum PCR and VII (b) marks patients studied by whole blood PCR in chapter VII.

Patients are ordered according to acute and then chronic graft versus host status (AGVH, CGVH) and then by active CMV status (by serum and/or urine PCR, buffy coat and/or urine DEAFF or culture).

Patient 1 II, III, VII (a)

This 34 year old man underwent allogeneic bone marrow transplantation for multiple myeloma. Acute graft versus host disease developed. CMV serology was donor negative/recipient positive. Despite ganciclovir prophylaxis, active CMV infection commenced on day 67 and therefore occurred concomitantly with the onset of symptoms (pyrexia) but 25 days before diagnosis of CMV pneumonitis. Ganciclovir therapy was initiated on day 67 but this patient did not respond initially and died 126 days posttransplant; interestingly this patient apparently cleared CMV 19 days before death.

See figure 3.4.
Patient 2  II, III, VII (a)
This 34 year old man underwent 'volunteer unrelated (VUD)' bone marrow transplantation for multiple myeloma. Acute graft versus host disease developed. CMV serology was donor negative/recipient positive. Serum PCR for CMV was initially positive on day 45 which was 85 days before symptoms (day 130; CMV retinitis). This patient appeared to clear CMV but urine PCR and DEAFF positivity was detected on day 100 until day 118 and CMV retinitis was diagnosed on day 130; CMV was subclinical until the diagnosis of CMV retinitis. This patient responded clinically to ganciclovir treatment (initiated on day 130) but died on day 309 from progressive multi focal leucoencephalopathy.

See figure 3.5.

Patient 3  II, III, VII (a)
This 44 year old man underwent allogeneic bone marrow transplantation for multiple myeloma. Acute graft versus host disease developed. CMV serology was donor negative/recipient positive; serum was initially positive on day 65 which was 6 days after the onset of symptoms (pyrexia). This patient cleared CMV after ganciclovir treatment from day 59 to day 66. Urine was not positive by PCR. This patient made a full recovery but died 10 months after transplantation from recurrent initial disease.

See figure 3.6.

Patient 4  II, III, VII (a)
This 41 year old man underwent 'volunteer unrelated ' (VUD) bone marrow transplantation. Acute graft versus host disease developed. CMV serology was donor positive/recipient negative and active CMV infection developed; urine positivity on day 102 was responded to initiation of full dose ganciclovir treatment the following day. CMV-symptoms were not observed. This patient died 9 months after transplantation from recurrent initial disease.

Patient 5  II, III, VII (a)
This 22 year old man underwent 'volunteer unrelated ' (VUD) bone marrow transplantation for acute lymphoblastic leukaemia. Acute graft versus host disease developed. CMV serology was donor negative/recipient positive but active CMV infection did not develop. However, this patient died 3 months after transplantation from gastrointestinal bleeding.
Patient 6 II, III, VII (a)
This 41 year old woman underwent allogeneic bone marrow transplantation for acute myeloid leukaemia. Acute graft versus host disease developed. CMV serology was donor negative/recipient positive but active CMV infection did not develop. This patient survives at 39 months after transplantation.

Patient 7 II, III, VII (a)
This 26 year old man underwent allogeneic bone marrow transplantation for acute lymphoblastic leukaemia. Acute graft versus host disease developed. CMV serology was donor positive/recipient negative but active CMV infection did not develop. This patient survives at 16 months after transplantation.

Patient 8 II, III, VII (a)
This 24 year old man underwent allogeneic bone marrow transplantation for acute lymphoblastic leukaemia. Acute graft versus host disease developed. CMV serology was donor negative/recipient negative and active CMV infection did not develop. This patient died 3 months after transplantation from Aspergillus pneumonia.

Patient 9 II, III, VII (a), VII (b)
This 39 year old woman underwent allogeneic bone marrow transplantation for chronic myelogenous leukaemia. Acute and chronic graft versus host disease developed. CMV serology was donor negative/recipient positive but active CMV infection did not develop. This patient survives at 30 months after transplantation.

Patient 10 II, III, VII (a)
This 48 year old man underwent allogeneic bone marrow transplantation for chronic myelogenous leukaemia. Acute and chronic graft versus host disease developed. CMV serology was donor positive/recipient positive but active CMV infection did not develop. This patient survives at 34 months after transplantation.

Patient 11 II, III, VII (a), VII (b)
This 52 year old woman underwent allogeneic bone marrow transplantation for acute myeloid leukaemia. Acute and chronic graft versus host disease developed. CMV serology was donor positive/recipient negative but active CMV infection did not develop. This patient survives at 32 months after transplantation.

Patient 12 II, III, VII (a)
This 41 year old man underwent allogeneic bone marrow transplantation for myelofibrosis. Acute and chronic graft versus host disease developed. CMV serology was donor positive/
recipient negative but active CMV infection did not develop. This patient survives at 40 months after transplantation.

**Patient 13 II, III, VII (a), VII (b)**
This 53 year old woman underwent allogeneic bone marrow transplantation for chronic myelogenous leukaemia. Acute and chronic graft versus host disease developed. CMV serology was donor negative/recipient negative and active CMV infection did not develop. This patient survives at 44 months after transplantation.

**Patient 14 II, III, VII (a)**
This 37 year old woman underwent volunteer unrelated (VUD) bone marrow transplantation for chronic myelogenous leukaemia. CMV serology was donor positive/recipient positive but the post-transplant course was not complicated by active CMV infection. This patient died after 3 months posttransplant from pulmonary haemorrhage.

**Patient 15 II, III, VII (a), VII (b)**
This 59 year old woman underwent allogeneic bone marrow transplantation chronic myelogenous leukaemia. CMV serology was donor positive/recipient negative but the post-transplant course was not complicated by active CMV infection. This patient survives at 31 months after transplantation.

**Patient 16 II, III, VII (a), VII (b)**
This 39 year old man underwent allogeneic bone marrow transplantation for chronic myelogenous leukaemia. CMV serology was donor negative/recipient negative and active CMV infection did not develop. This patient survives at 33 months after transplantation.

**Patient 17 II, III, VII (a)**
This 37 year old woman underwent allogeneic bone marrow transplantation for acute myeloid leukaemia. CMV serology was donor negative/recipient negative and active CMV infection did not develop. This patient survives at 42 months after transplantation.
## Appendix 3  In Situ Hybridisation and Immunohistochemistry for Active CMV (see Chapter 6)

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<th>Patient</th>
<th>Group</th>
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Appendix 3 gives full data for the patients (numbered) studied in Chapter 6. CMV antibody status (Ab. status) is shown for donor (D) and recipient (R) and detection of active CMV infection is indicated (+ active CMV detected, - active CMV not detected). HLA mismatching (+ indicates mismatch, - indicates partial or complete match) is given for HLA alleles A, B and DR. The results generated by immunohistochemistry and in situ hybridisation are given; results are shown for hepatocytes (Hep.), mononuclear cells (Mono.), epithelial cells (Epi.) and endothelial cells (End.) and + indicates CMV positive cells identified. Graft loss occurred following chronic rejection (CR), hepatic artery thrombus (HAT) or other diseases (other). The latter comprised hepatitis C related cirrhosis (*1), recurrent disease (*2) and unspecified biliary tract disease (*3). Fourteen grafts were retained (retained).
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