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**INVESTIGATIONS INTO THE PATHOGENESIS OF HUMAN
CYTOMEGALOVIRUS**

A thesis submitted to the University of London
for the degree of

Doctor of Philosophy

in the Faculty of Medicine

by

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ABSTRACT

Human cytomegalovirus (HCMV), a member of the betaherpesviruses is a major cause of morbidity and mortality in the immunocompromised host. However, the pathogenesis of HCMV disease still remains unclear. This thesis has investigated the pathogenesis of HCMV in the immunocompromised host. Quantitative polymerase chain reaction (PCR) methods were used for the longitudinal measurement of HCMV viral load in congenitally infected infants and recipients of renal and liver transplants. The association between HCMV viral load and HCMV disease in the context of other risk factors for disease and response to antiviral therapy was examined.

Measurement of HCMV load was found to provide important prognostic information and allowed the effects of antiviral therapy on virus clearance to be assessed. Elevated viral load was significantly associated with risk of HCMV disease in renal and liver transplant recipients ($p < 0.01$ and $p < 0.0001$, respectively). In addition, elevated HCMV viral load remained an independent risk factor following multivariate analysis in both transplant populations. In contrast, the risk of donor seropositivity was negated after controlling for HCMV viral load in both renal and liver transplant recipients indicating that this is the mechanism through which this risk factor operates. The administration of augmented methylprednisolone for rejection episodes in liver transplant recipients was also identified as an independent risk factor for disease in liver transplant recipients and acted to reduce the viral load level required to produce disease.

The dynamics of anti-gB antibody levels in liver transplant recipients was investigated. In these individuals, the timing and level of specific anti-gB antibodies was significantly associated with the level and timing of appearance of HCMV DNAemia.

Finally I developed and optimised a fully quantitative reverse transcriptase PCR assay (QCRT-PCR) for the detection of HCMV gB mRNA with a view to providing further information into the dynamics of HCMV gB expression and in monitoring antiviral efficacy.

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ABBREVIATIONS

aa	amino acid
AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
AZT	3' azidothymidine
bid	bis die (twice a day)
bp	base pairs
BSA	bovine serum albumin
cDNA	complimentary DNA
CDC	Centers for Disease Control
CDV	cidofovir
CMV	cytomegalovirus
CNS	central nervous system
cpe	cytopathic effect
CPM	counts per minute
CSF	cerebrospinal fluid
CTL	cytotoxic T-lymphocyte
ddC	dideoxycytidine
ddI	dideoxyinosine
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
EBV	Epstein-Barr Virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoabsorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
gB	glycoprotein B
GCV	ganciclovir
gH	glycoprotein H
GID	gastrointestinal disease
gp	glycoprotein
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HIV	Human Immunodeficiency Virus
HLA	Human leucocyte antigen
HSV	Herpes simplex virus
IC ₅₀	minimum inhibitory concentration for 50%
IE	immediate early
IgG	Immunoglobulin
IT	induction therapy
iv	intravenous
kb	kilobase
LTR	long terminal repeat
M	matrix protein
mAb	monoclonal antibody
MCP	major capsid protein
mCP	minor capsid protein
MHC	major histocompatibility complex
MIE	major immediate early
min	minute
moi	multiplicity of infection

Mr	relative molecular mass
mRNA	messenger ribonucleic acid
NK	natural killer
OD	optical density
o.d	omni die (once a day)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBL	peripheral blood leukocyte
PBS	phosphate buffered saline
PCP	<i>Pneumocystis carinii</i> pneumonia
PCR	polymerase chain reaction
PFA	foscarnet
pfu	plaque forming unit
pi	post-inoculation
PM	post mortem
po	per oram (orally)
pp	phosphoprotein
QCPCR	quantitative competitive PCR
QCRT-PCR	quantitative competitive reverse transcriptase PCR
q.d.s	quater die sumendus (four times a day)
RNA	ribonucleic acid
RNAse	ribonuclease
RT	reverse transcriptase
SD	standard deviation
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SEM	standard error of the mean
<i>Taq</i>	<i>Thermus aquaticus</i>
t.d.s	ter die sumendus (three times a day)
TE	Tris-EDTA
UK	United Kingdom
USA	United States of America

Chapter 1.

General Introduction Human Cytomegalovirus (HCMV)

1.0 Human Cytomegalovirus Characterisation

1.1 Classification

Human Cytomegalovirus (HCMV) is classified as a member of the *Herpesviridae* family of viruses. There are three subfamilies within this group, alpha (α), beta (β) and gamma (γ) *herpesvirinae*. HCMV is characterised as a β -*herpesvirus* due to its slow growth in fibroblasts *in vitro* together with genetic differences (McGeoch *et al*, 1995). Cytomegaloviruses are highly species specific although one recent report has shown that baboon CMV can replicate in human fibroblast cells (Michaels *et al*, 1997). Restriction enzyme digestion of virion DNA has revealed differences in the genetic composition of the virus, which can lead to variation in antigenic constitution. These genetic differences do not constitute sub-types since the host immune response can recognise a variety of strains within the same individual.

1.1.2 Virion Morphology

HCMV is an enveloped virus with icosahedral symmetry approximately 150-200 nm in size and has typical herpesvirus-like morphology when viewed by electron microscopy (figure 1.1). The DNA is contained within a viral capsid composed of 162 hexagonal capsomeres which is in turn surrounded by an abundant tegument protein layer associated with an outer lipid envelope which contains a number of virally encoded glycoproteins. When the virus is cultured *in vitro* two additional particles can be detected; dense bodies, composed of the viral envelope and tegument only, and non-infectious enveloped particles (NIEP's) composed of a lipid envelope with a capsid devoid of DNA.

1.1.3 Genome Organisation

The genome of HCMV is made up of a complex arrangement of unique and repeat sequences. HCMV has the largest of the herpesvirus genomes consisting of linear double stranded DNA 229,354 base pairs (bp) long, which can be divided into two unique components, the unique short region (US) and the unique long region (UL) a schematic representation is shown in figure 1.2.

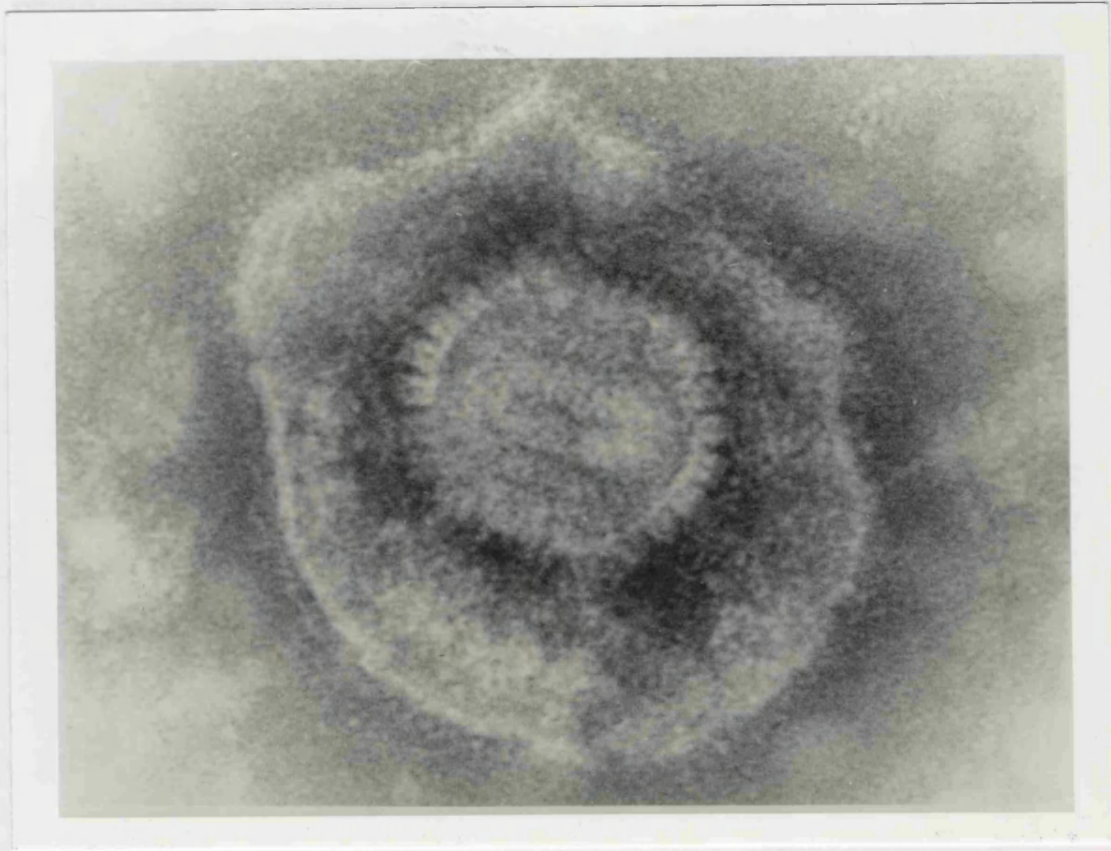


Figure 1.1 Electron micrograph of Human Cytomegalovirus (HCMV).

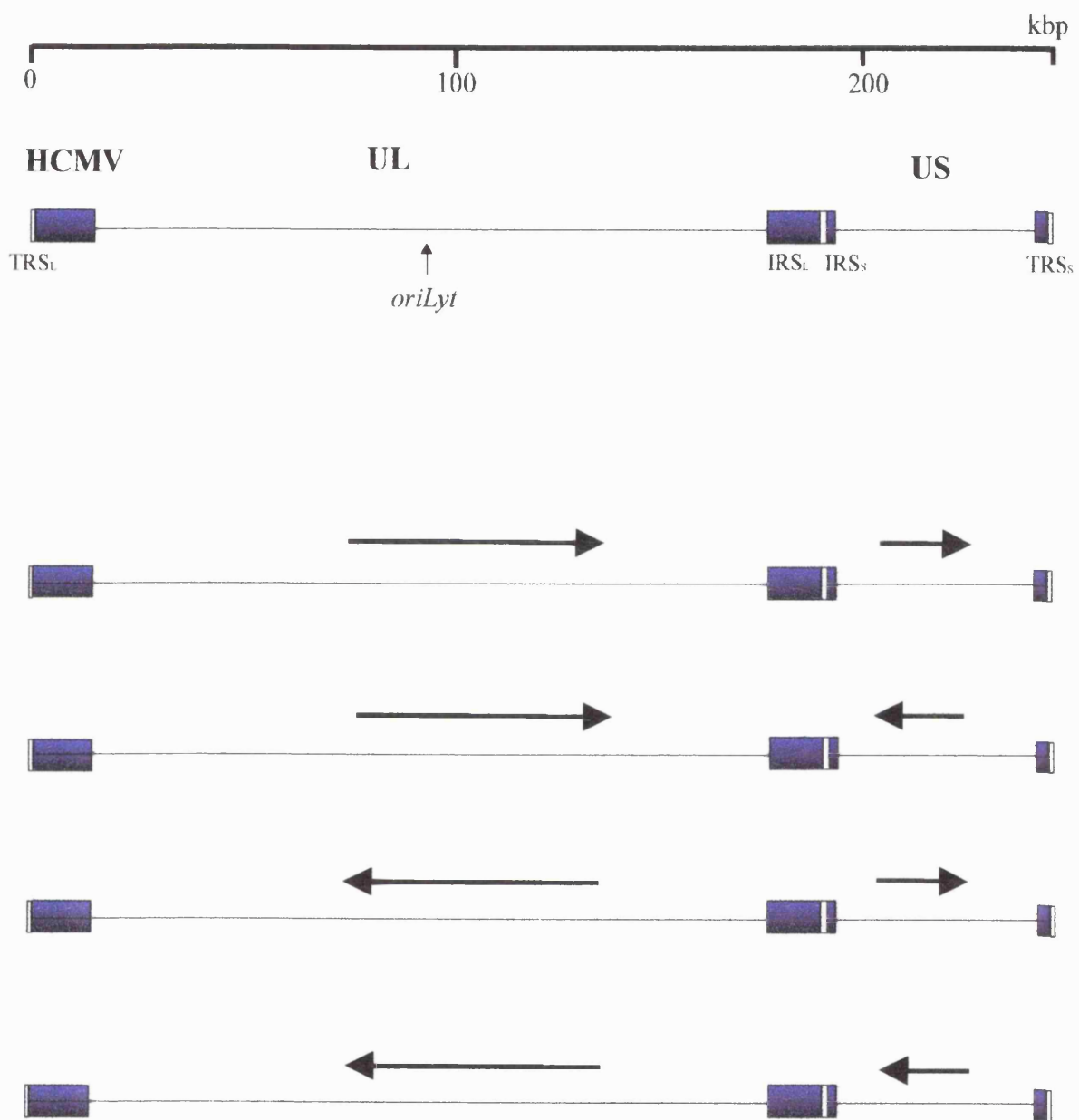


Figure 1.2 Schematic representation of the genome organisation of HCMV.

The UL (169, 972bp long) region is flanked by two repeat sequences, namely an internal (IR_L) and a terminal repeat sequence (TR_L), while the US (35,418 bp long) region is flanked by two repeats termed IR_S and TR_S. Inversion of the UL or US component of the genome results in four isomeric forms of the virus which exist in equimolar quantities in culture. The G+C overall content is relatively high (57.2%) although there is considerable variation in the distribution in G+C throughout the genome. The less virulent HCMV laboratory strain AD169 has been sequenced in its entirety, and sequence analysis revealed a total of 208 predicted open reading frames (Chee *et al*, 1990). The “prototype” sequence of AD169 has formed the basis of sequence analysis comparisons to viral isolates from patient material. However, it is important to note that AD169 is a laboratory adapted strain which has been subjected to multiple passage in culture, thus it may not be entirely representative of the virus strains that exist *in vivo*. Indeed, Cha *et al* (1996) noted that some 22 open reading frames present in clinical isolates were not found in Toledo laboratory strains of the virus. These strains can still be efficiently propagated in culture although their pathogenicity remains to be elucidated.

The enzymatic digestion of genomic DNA from clinical isolates using restriction endonucleases shows unique restriction fragment length polymorphism patterns (RFLP's). The identification of different RFLP's in different individuals has led to important information into the pathogenesis of HCMV in the immunocompromised host (Winston *et al*, 1985, Grundy *et al*, 1988). The majority of sequence variations between different RFLP's are silent point mutations located in the UL and US region of the genome, although mutations have been found in the junction of the UL and US regions of the genome (Weststrate *et al*, 1983). Indeed, different incoming strains from donors in renal transplant patients were identified in patients at risk of either reactivation or reinfection, which were associated with an increased risk of HCMV disease compared to patients at risk of reactivation of latent virus (Grundy *et al* 1988). In contrast, in bone marrow transplant recipients risk of HCMV disease was primarily associated with reactivation of latent endogenous virus strains (Winston *et al* 1985).

1.1.4 Proteins

HCMV has the largest genome of the herpesviruses with 208 open reading frames. However, only 202 proteins are encoded by these open reading frames due to a series of complex splicing events, and of these, 178 proteins are unique due to more than one copy of the gene being mapped to a repeat sequence within the genome (Chee *et al*, 1990, reviewed by Griffiths and Emery, 1996). Nomenclature for the proteins is

standardised; the prefix p denotes protein, gp glycoprotein and pp phosphoprotein, followed by a number to identify the genetic locus within the UL or US region and any other trivial name is placed in brackets. Figure 1.3 shows a schematic representation of the proteins identified to date together with their genetic locus (kindly provided by Griffiths and Emery 1998).

There are approximately 30 genes present in the UL region conserved amongst all eight human herpesviruses. The proteins of HCMV can be generally grouped into the structural proteins that make up the virion structure and non-structural proteins or enzymes that are found in the infected cell and are responsible for viral replication. The structural proteins fall into three main categories, capsid proteins, tegument proteins and envelope proteins. The non-structural proteins can be grouped into transactivators, G-coupled receptor proteins and replication enzymes that may be virion associated. For the purpose of this thesis only a brief description of the major proteins of HCMV and their function follows: a more detailed description can be found by Mocarski (1996, Fields 3rd edition).

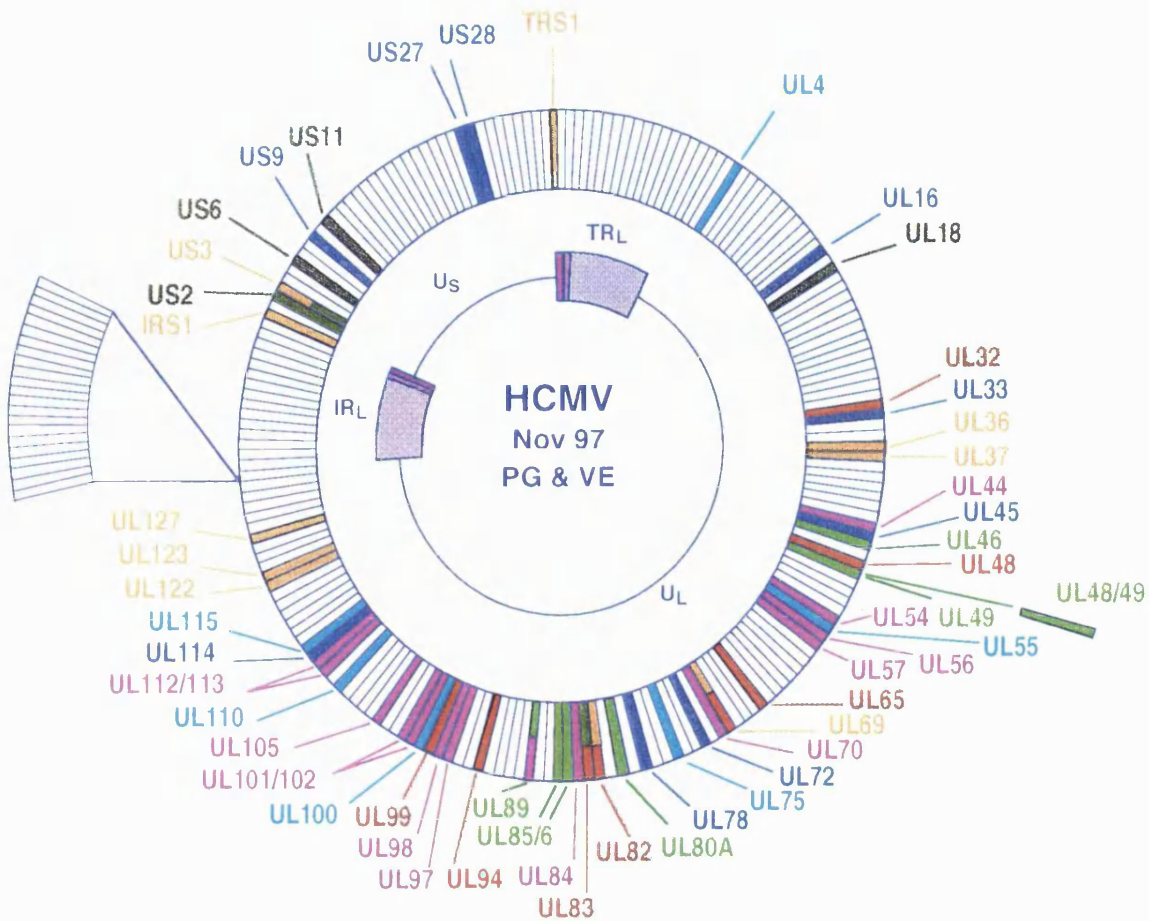
1.1.5 Structural proteins

1.1.5.1 The capsid proteins

The major capsid protein or MCP (pUL86) shows low immunogenicity in humans, a high degree of homology and cross reaction with VP5 of herpes simplex virus (HSV) (Jahn *et al*, 1990). MCP also shares common epitopes with the gag p55 gene of HIV. The minor capsid protein or mCP (pUL46) shares distinct homology with VP19C of HSV. pUL80a is derived from the carboxyl terminal half of the gene pUL80 which possesses a protease embedded in the amino terminus of the protein and undergoes autocatalytic activity to cleave the molecule at three positions. The pUL80a gene product is cleaved at the amino terminus to produce a protease and an assemblin protein and shows striking similarity to the VP22a of HSV. The cleavage of the large assembly protein precursor of VP22a is involved in DNA packaging and the same function is proposed for pUL80a of HCMV (Hall *et al*, 1997).

1.1.5.2 Tegument proteins

These proteins are predominately phosphoproteins that lie in between the viral capsid and the virion envelope. The most abundant of the tegument structural proteins are the basic phosphoprotein pp150 (ppUL32) and the lower matrix protein pp65 (ppUL83) both of which produce a dominant immunological response in humans and consequently are










Colour Coding	
	= Tegument
	= Transactivators
	= DNA replication
	= Capsid & assembly
	= Envelope
	= Others
	= Immune evasion

Figure 1.3 Localisation of genes encoding the proteins that constitute the HCMV genome (Kindly provided by Griffiths and Emery 1998).

commonly used as the basis of serological tests (Gerna *et al*, 1991, Landinni *et al*, 1990 & 1995, Ehmst *et al*, 1993). The lower matrix protein is the predominant protein found in dense bodies and can constitute up to 95% of the total protein component. Indeed, an abundance of pp65 antigen can be detected in the peripheral blood of immunocompromised patients with active HCMV infection (Grefte *et al*, 1994). However, mRNA of the pp65 transcripts was not detected in these cells suggesting a phagocytic uptake of this protein by leukocytes as opposed to *de novo* synthesis. This protein is a major target for the cytotoxic T-cell immune (CTL) response in humans (McLaughlin-Taylor *et al*, 1994, He *et al*, 1995, Bopanna *et al*, 1996a, Wills *et al*, 1996). The upper matrix protein or pp71 (UL82) shares a high degree of sequence homology with pp65 but, unlike pp65 is considerably less abundant and acts in concert with pUL69 as a potent transactivator of gene expression. pp71 transactivates promoters with ATF (activation transcription factor) or AP-1 (activator protein) binding sites and is analogous to the α -transinducing factor VP16 of HSV despite a lack of homology at the amino acid level. There are two other main phosphoprotein components of the tegument, namely pp28 (UL99) and pp130 (UL56). The former is highly immunogenic and localises at the capsid surface, the latter is homologous to the ICP18.5 gene of HSV and is involved in virion maturation.

1.1.5.3 Envelope proteins

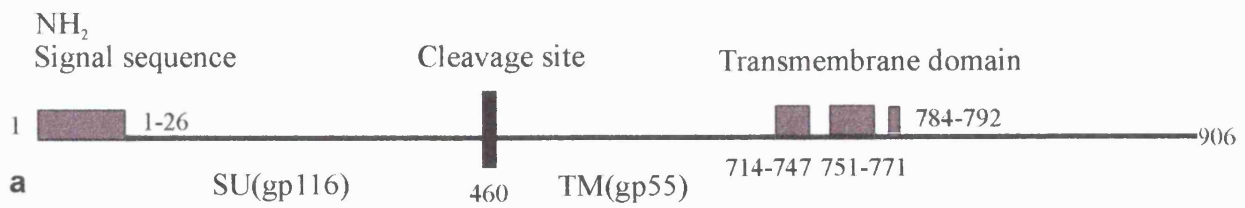
The virion envelope comprises host lipids and viral proteins and is derived from either the inner nuclear or cytoplasmic membranes. The main virus derived constituents of the virus envelope are glycoproteins. Approximately 55 ORF's coding for the glycoproteins have been identified by sequence analysis of AD169 strain of HCMV (Chee *et al*, 1990). Homologues of four major glycoproteins of HSV have been fully characterised using null mutants (gB, gH, gL and gM). Despite the existence of many other glycoproteins and their identification, the function of these proteins in the replicative cycle of the virus remains to be fully elucidated. For the purpose of this thesis, a brief description of the main glycoproteins of HCMV and their functions are detailed below, a comprehensive review can be found by Britt *et al* (1996).

The most abundant of the envelope glycoproteins that constitutes up to 50% of the total protein mass is the highly conserved glycoprotein B or gB (UL55). gB shares sequence homology with the alpha-herpesviruses HSV, varicella zoster virus (VZV) pseudo rabies virus (PRV) and the beta-herpesviruses MCMV, HHV-6, HHV-7. Glycoprotein B is a transmembrane protein existing as a complex within the virion envelope. It is composed

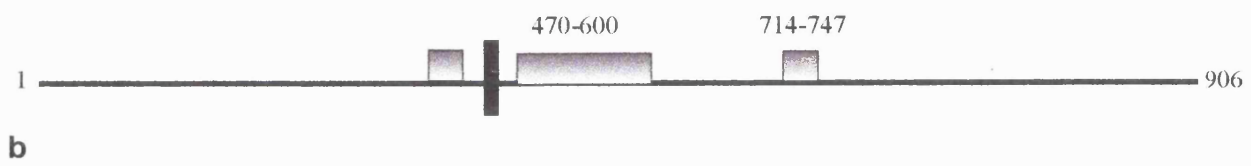
of two components linked together by disulphide bonds, gp58 the transmembrane component and gp116 the surface subunit. The protein is transcribed as an early-late protein which accumulates late in infection and has been implicated in membrane penetration, fusion of infected cells and transmission from cell to cell (Tugizov *et al*, 1994, 1995). Glycoprotein B forms the major target for neutralising antibody response in human sera as well as being a target for CTL responses identifying this protein as a potential source for recombinant based sub-unit vaccines (Britt *et al*, 1990, Gonczol *et al*, 1991, Kneiss *et al*, 1991, Liu *et al*, 1991 & 1993, Riddell *et al*, 1991, Marshall *et al*, 1994, Beninga *et al*, 1995). Two major linear neutralising epitopes and four additional conformational epitopes have been identified illustrating the complexity of the gB molecule. A schematic representation of the localisation of the epitopes is shown in figure 1.4. The first of the linear epitopes AD-1 located in the gp58 portion of the molecule between amino acids 552-635 and elicits the majority of the neutralising antibody response in human sera, while AD-2 resides in the amino terminus of gp116 between amino acids 68-77. Three of the conformational epitopes lie in the central region of the protein, two of which overlap AD-1, while the fourth domain is located in the extracellular carboxyl terminal region of the protein.

Another important glycoprotein component of the virion envelope involved in the humoral immune response is glycoprotein H or gH (gpUL75). Although less abundant than gB, gH has been reported to elicit approximately 30% of the total neutralising activity in human sera. One report has shown antibodies to gH to be important in limiting HCMV retinitis in patients with AIDS (Rasmussen *et al* 1994). The gene is highly conserved with approximately 96% conservation at the amino acid level amongst different strains (Chou *et al*, 1991). This late gene has been shown to have a role in membrane fusion in the initial stages of infection (Keay *et al*, 1991, Rasmussen *et al*, 1991a). Glycoprotein H forms a complex with another glycoprotein component of the envelope, gL (gpUL115), analogous to the gH/gL complex of HSV, and is essential for transport of the molecule to the cell surface (Speate *et al*, 1993). Like gB, gH elicits both humoral and cellular immune responses and recombinant gH has been shown to induce CD4+ T -cell proliferative responses (Beninga *et al*. 1995). One linear neutralising epitope has been identified at the amino terminus of the protein between amino acids 34-43 which is strain specific and absent in the laboratory strain Towne neutralising epitope has been identified at the amino terminus of the protein between amino acids 34-43 which is strain specific and absent in the laboratory strain Towne In addition, three conformational neutralising epitopes have been reported whose precise location has not been identified, however,

Structural Features of gB(UL55)



Proposed functional domains of gB(UL55)



Antigenic domains of gB(UL55)

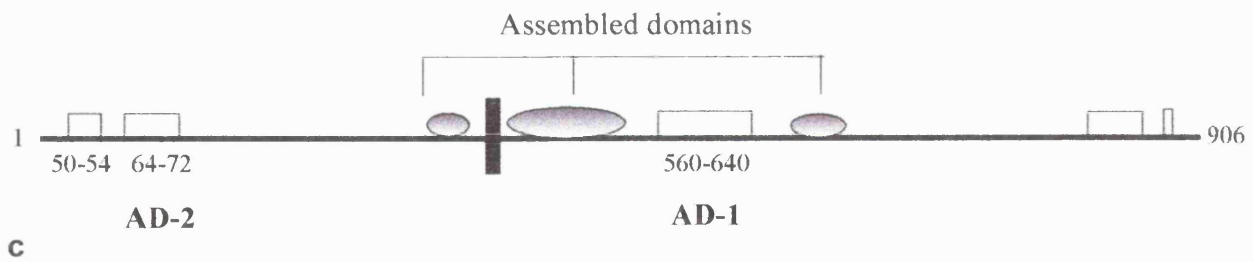


Figure 1.4 Localisation of the two linear (AD-1 & AD-2) and three conformational epitopes of HCMV gB (assembled domains) not drawn to scale taken from Britt *et al*, 1996. SU (surface subunit), TM (transmembrane).

binding of antibody is abrogated upon deletion of the carboxyl terminus of the protein (Zheng *et al*, 1996).

The integral membrane protein gM (UL100) forms part of the glycoprotein complex II or gCII 2 (gp47-52) and shows sequence homology to the non-essential gM protein of HSV. gM has multiple membrane spanning domains as well as heparin binding activity due to its association with the gCII complex (Kari *et al*, 1992). gCII 1 of the glycoprotein complex is made up of a group of proteins located in the US6-11 region of the genome which are dispensable for growth *in vitro* (Jones *et al*, 1991). The precise role of these glycoproteins in the structure of the mature complex is unclear, and transcription involves an intricate series of splicing events and multiple use of alternative transcription initiation sites. Interestingly, the products of US6 and US11 have recently been shown to interfere with class I MHC antigen presentation as will be discussed further in section 1.6.3 (Wiertz *et al*, 1996).

1.1.6 Non-structural proteins

1.1.6.1 Transactivators

Several genes of HCMV are able to transactivate both homologous and heterologous promoters. The major immediate early (MIE) complex encoded by UL122/123, the most extensively studied area of the genome responsible for gene regulation, is made up of a strong promoter/enhancer region. Differential complex splicing events leads to three isoforms following infection. The first of the isoforms is referred to as IE1 (IE72) or immediate early 1 a Mr 72,000 protein, the second IE2 (IE86, Mr 86,000) and the third is a Mr 55,000 protein (IE55) which is an isoform of IE2. A total of five proteins to date have been mapped to the IE1/IE2 region, four are expressed at immediate early times and the fifth is expressed a late times. IE1, the most abundant protein, accumulates in the nucleus of infected cells following infection and is a product of the UL123 gene, whereas IE86 is derived from UL122 accumulating at later times during infection. *In vitro* transcription studies revealed that the IE1 gene is spliced and contains 4 exons, IE2 is also spliced and shares the first three exons of IE1.

Upstream of the MIE complex is an enhancer region which is important for the initial binding of the transcription factors AP1 and ATF which are presented in association with the tegument proteins UL82 and UL69 following infection (Meier *et al*, 1996). Expression of the MIE complex then proceeds without any prior DNA replication, IE1 activates the MIE promoter while IE2 acts as an auto repressor. Both interact synergistically via NF κ B sites

to drive the enhancer further. The repressor function of IE2 is thought to occur by direct binding to *crs* repressor sequence which is located within the CAP site of the MIE promoter itself (Cherrington *et al*, 1991, Liu *et al*, 1991). The isoform IE55 is expressed transiently at early times *in vitro* and differentially according to cell type (Barrachini *et al*, 1992, Kerry *et al*, 1996). This protein is able to activate the MIE promoter as well as abrogate the repressor function of IE2. Differential expression of IE55 according to cell type may be related to the physiological environment of the cell and may subsequently lead to altered regulation of the MIE complex and thus levels of protein expression, which may in turn alter viral replication (Kerry *et al*, 1996, Meier *et al*, 1997). The modulator sequence of the MIE gene complex does not appear to be important in controlling the mechanism for regulating expression in differentiated and undifferentiated cell types (Meier *et al*, 1997).

Both IE1 and IE2 are required to activate the early promoters of the DNA polymerase gene (UL54) and the lower matrix protein pp65. IE2 alone can activate the early promoters of UL4 and UL112/113. The process involved in the activation of these early promoters is very complex and can be mediated by other early genes such as UL36-38 and IRS1/TRS1 (Pari *et al*, 1993, Iskenderian *et al*, 1996). Indeed, the gene products of these aforementioned proteins act in synergy with IE1/IE2 to activate early promoters. UL112-113 proteins are able to transactivate early promoters of HCMV in a promoter dependent manner. In contrast, the gene products of UL36-38 and IRS1/TRS1 in association with IE1/IE2 are promiscuous in their ability to transactivate several promoters within the HCMV genome.

1.1.6.2 Homologues of cellular gene products

HCMV encodes a number of protein homologues. One such protein is gpUL18 which has been identified as the MHC class I homologue and may be important in immune evasion by HCMV as discussed later in section 1.6.3. The UL18 gene is non-spliced in contrast to its host cell counterpart which is comprised of eight exons and seven introns. *In vitro* experiments have revealed that in virus infected cells there is a marked reduction in MHC class 1 (Wiertz *et al*, 1996, Machold *et al*, 1997). Later studies revealed that UL18 alone was not responsible for class I down regulation and is dispensable for growth of the virus *in vitro*. In addition other components of the genome namely the US2, US11 region contribute to the down regulation of class I (Jones *et al*, 1995).

Also included in this category are a group of proteins that are homologous to the G-

coupled protein receptor proteins or GCR's encoded by UL33, UL78, US27 and US28 (Welch *et al*, 1991, Rawlinson *et al*, 1996). These genes are transcribed at late times during infection and are thought to play a role in virus interaction with different cell types during infection. The US28 gene shares approximately 30% homology with mammalian leukocyte receptors for α and β chemokine receptors. US28 encodes a C-C chemokine receptor and specifically binds monocyte chemoattractant protein 1 (MCP1), MIP1 α , MIP1 β and RANTES (Neote *et al*, 1993). The precise function remains to be fully elucidated, however, the proposed mechanism postulates that the binding of US28 increases intracellular calcium levels to provide a suitable environment for activation of the immediate early promoter and thus viral gene expression.

1.1.6.3 Other proteins

There are proteins that are virion associated that do not necessarily fall into the category of structural proteins but are important in the life cycle of the virus during infection. Only two of these proteins relevant to this thesis are described below. The advent of antiviral drugs for controlling HCMV infection has led to the recognition of the importance of the pUL97 gene which encodes a protein kinase (Littler *et al*, 1992). Initial marker transfer studies identified two domains within the HCMV genome that were responsible for resistance to the antiviral drug ganciclovir, one located within the DNA polymerase gene (UL54) and the other mapped to the UL97 region of the genome (Sullivan *et al*, 1992, Smith *et al*, 1997). The UL97 gene shows functional homology to the thymidine kinase (Tk) gene of HSV and VZV in so far as it phosphorylates both aciclovir and ganciclovir (Sullivan *et al*, 1992, Erice *et al*, 1997). However, unlike HSV and VZV, the role of UL97 in the replicative cycle of the virus still remains to be fully elucidated. The UL97 gene of HCMV is able to phosphorylate ganciclovir into the monophosphate form of the drug, which is subsequently activated by cellular enzymes to the active triphosphate form, where it acts to inhibit HCMV DNA polymerase as will be discussed later in section 1.10.

1.2 HCMV Replication

1.2.1 Gene Regulation

The genes of HCMV are transcribed as an ordered cascade of events analogous to that first observed for HSV by Roizman *et al* (1974). Initially the alpha or immediate early genes (α , IE) are transcribed followed by transcription of the early or beta (β , E) genes and finally the gamma or late (γ , L) genes are transcribed. The transcription of the IE genes is mediated by the host RNA polymerase II (RNA-pol II) and does not require any

prior viral DNA synthesis. The IE genes are the first to be expressed following infection and map to the UL122/123 region of the genome as described in section 1.1.6. The MIE promoter is activated by initial binding of host transcription factors that are recruited by the synergistic interaction of pUL69 and pUL82. Viral mRNA for the early proteins is then transcribed, which leads to DNA replication followed by the expression of the late genes. The virion is finally assembled and the new virus particles are released from the host cell.

During infection of the host cell, several cellular genes are also activated including DNA and RNA polymerases, plasminogen activator and DNAses, presumably to create a suitable environment for viral replication. HCMV infection causes an arrest in cell cycle progression at the G₁ and G₂/M phase (Jault *et al*, 1995, Brensnahan *et al*, 1996, Lu *et al*, 1996). Cyclin A and D are not induced, however, the levels of cyclin E and p53 are increased. There is some debate over the levels of cellular DNA expression, Jault *et al* (1995) reported an increase in the levels of cellular DNA synthesis, however, Brensnahan *et al* (1996) reported that cellular DNA synthesis was shut down. It is clear from these studies that cellular proliferation and the cell cycle are arrested in order to provide a favourable environment where all the precursors for viral replication are available.

1.2.2 HCMV DNA Replication

HCMV DNA synthesis is delayed compared to that of HSV, where peak DNA synthesis is detected 1-3 days post infection. In tissue culture plaques typically appear 7-14 days post-infection. DNA is synthesised by rolling circle replication to produce DNA concatamers which are cleaved into linear unit length genomes and subsequently packaged into virions. There are many non-structural genes involved in this process which utilise a variety of promoters, the first and most important of these regulatory genes to be transcribed is the MEI complex which provides the starting point upon which all subsequent events are based. Indeed, mutants lacking the IE1 gene of HCMV have a severely impaired ability to replicate *in vitro* (Mocarski *et al*, 1996, Greaves *et al*, 1998).

Requirements for DNA synthesis include a replication complex encoded by a variety of essential proteins and a defined initiation site or *cis*-acting element referred to as *ori*-Lyt. The *ori*-Lyt origin of replication for lytic replication of HCMV is located in the centre of the UL region of the genome upstream of UL57. Eleven essential genes are required for *ori*-Lyt mediated replication, all located in the central region of the UL portion of the genome and consists of; a two subunit DNA polymerase or Pol encoded by UL44 and UL54, a helicase-primase complex encoded by the genes UL70, UL102, UL105 and a single

stranded DNA-binding protein encoded by UL57 (Pari *et al*, 1993). The replication fork genes by virtue of their exclusive identity with the viral genome, have formed the basis of anti-HCMV drug design as will be discussed further in section 1.10. The polymerase gene, identified due to its homology with the HSV, has 3'→5' proof-reading exonuclease activity and presumed 5'→3' exonuclease and RNase H activity (Marcy *et al*, 1990). The polymerase associated or accessory protein (UL44) a nuclear DNA-binding phosphoprotein that shares homology with HSV analogue ICP36, physically associates with UL54 and stimulates polymerase activity (Ertl *et al*, 1991&1992, Weiland *et al*, 1994). The helicase-primase complex unwinds the template at the replication fork and the primase activity initiates lagging strand DNA synthesis. The UL57 ORF encodes the single stranded DNA-binding protein and shares homology with the HSV-1 major DNA binding protein (UL29 or ICP8). It is assumed that UL57 of HCMV stabilises and renders the single stranded DNA produced as a result of the helicase, available to the polymerase in a manner analogous to that of ICP8. The other regulatory genes involved in DNA replication are US36-38, UL122/123 (MIE region) and IRS1/TRS1. UL84 was recently discovered to bind to and modulate IE2 by auto regulation (Gebart *et al*, 1997). UL112/113 was found to bind to and activate the UL54 promoter in association with other MIE proteins (Kerry *et al*, 1996). The UL89 ORF was recently identified as a component of the terminase complex involved in packaging and cleavage of concatamers (Underwood *et al*, 1998). This study analysed the effects of BDCRB (2-Bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole) a new inhibitor of DNA maturation on virus infected cells. Resistance to this compound was mapped to the UL89 ORF since BDCRB prevents viral DNA maturation by interacting with UL89 of HCMV.

1.2.3 Virus Packaging

Once DNA replication has been completed and after the expression of the late proteins, assembly and packaging of the viral DNA into mature virions occurs. Initially nucleocapsid structures accumulate in the nucleus, which can be observed under light microscopy as typical “owls eye” inclusion bodies. The DNA is thought to be packaged prior to encapsidation in the nucleus. The immature viral particles bud through the nuclear membrane into the perinuclear space, acquiring the envelope glycoprotein and tegument constituents. The particles then move from the outer nuclear membrane into cytoplasmic vesicles. Final envelopment and egress from the host cell is thought to be mediated by early endosomal compartments (Tooze *et al*, 1993). It is suggested that particles lose and then re-acquire the envelope from the endoplasmic reticulum during egress from the vesicles. The virion may also be transported in vesicles through the Golgi network to the

cell surface in an exocytic manner.

Mature virions are then released from the cells into the extracellular space at approximately 120 hours post-infection. In addition to the mature virions, defective viral particles can also be detected during infection *in vitro*. Dense bodies are composed of the viral envelope devoid of the capsid structure and viral DNA, containing primarily the tegument protein ppUL83 (pp65). Non-infectious particles (NIEP's) are indistinguishable from the mature virions but lack the viral DNA. The major structural components that make up the virion structure are the major and minor capsid proteins UL86 and UL46 respectively. The pUL80a ORF or assemblin encodes an assembly protein and a viral protease required for DNA packaging. The UL80a is cleaved at the amino terminus to produce the assemblin and serine protease. The three-dimensional structure of the protease has recently been shown to comprise a seven stranded β -barrel enclosed by a series of α -helices which constitute the carboxyl terminus of the enzyme (Qui *et al*, 1996, Shieh *et al*, 1996). The structure is unique when compared to the structure of known serine proteases such as chymotrypsin and subtilisin. Dimerisation of the molecule appears to effect activity and may confer specificity and recognition in substrate binding.

1.3 Epidemiology

Humans represent the only host that can be infected by HCMV. Transmission can occur indirectly or by direct person to person contact. The seroprevalance detected by the presence of IgG is geographically and economically dependent. In developing countries HCMV seroprevalance has been estimated to be approximately 80-90% of the adult population compared to 60% in the developed world. Transmission can occur either vertically or horizontally via numerous infectious bodily fluids such as saliva, urine, blood and semen. Primary infection, which usually occurs during infancy and less frequently during adolescence, is followed by persistence throughout life as viral latency. Intermittent recurrences are associated with shedding of the virus from various sites which perpetuates the virus throughout the population.

HCMV infection can be transmitted directly to the fetus as intrauterine infection or perinatally via the genital tract during birth or by breast feeding (Numazaki *et al*, 1997). Postnatal transmission is more difficult to define, however, the main routes of transmission are saliva or sexual contact. Primary infection rates amongst pregnant women vary according to socioeconomic conditions, where women of low income are

more susceptible to infection (Stagno *et al*, 1986). Day care centres have been identified as major sites of risk for infection in pregnant women from children shedding virus in urine and saliva due to sub-clinical infection (Hutto *et al*, 1985, Pass *et al*, 1985 & 1986, Shen *et al*, 1993a, 1993b).

Sexual transmission of the virus in the adult population represents the other main route of HCMV infection. The rates of viral shedding in the genital tract range from 3 - 35% but these figures can be misleading, since the majority of studies have been based on pregnant women attending STD clinics (Chandler *et al*, 1985). The rate of excretion in males is not clearly defined, although, high seroprevalence rates have been detected amongst patients attending STD clinics (Shen *et al*, 1994a). The frequency and quantity of viral shedding in homosexual men attending these clinics is greater than for heterosexual men (Mintz *et al*, 1983). In addition genital shedding of the virus is markedly increased in the sexually active population (Collier *et al*, 1995, Yang *et al*, 1995).

Post-natal primary infection by HCMV is asymptomatic in approximately 90% of cases especially in children, but in adults, the infection can manifest itself as a mononucleosis type syndrome which is often mistaken for Epstein Barr Virus (EBV) infection. Conversely in individuals with a suppressed immune system, due to either immaturity, organ transplantation or HIV infection, the risk of disease due to HCMV infection is considerably greater. The greatest risk of primary HCMV infection in transplant recipients is from the donor organ itself or to a lesser extent via blood transfusion. In the latter case, HCMV is thought to reside in cells as latent virus which then reactivates on encountering an allogeneic stimulus in the recipient (Winston *et al*, 1985, Soderberg-Naucler *et al*, 1997). In an attempt to reduce the risk of primary infection, blood products are screened for HCMV and where possible seronegative organs and blood products are transplanted into seronegative recipients. Therefore, the commonest route of HCMV infection in the immunocompromised host is due to recurrent infection either by reactivation of endogenous latent HCMV or by reinfection with exogenous virus.

1.4 Pathology and Pathogenesis

The virus can be detected in a variety of organs by histological and *in situ* hybridisation techniques. HCMV has also been detected in the leukocytes of circulating peripheral blood during HCMV infection (Gerna *et al*, 1991, Grefte *et al*, 1994, Meyer Konig *et al*, 1995). Indeed, removal of the leukocytes from the blood dramatically reduces transfusion mediated HCMV infection (Lang *et al*, 1977, Winston *et al*, 1980, Bowden *et al*, 1995,

Landaw *et al*, 1996). HCMV can infect a wide variety of cell types including epithelial, endothelial, and as recently discovered fibroblast cells, resulting in viral dissemination amongst a variety of tissues (Singzer *et al*, 1995). In addition, the parenchymal cells including neurones of the brain and retina, smooth muscle cells and hepatocytes are susceptible to infection. The diversity of cells that can be infected with HCMV leads to wide dissemination of the virus which can be detected at post-mortem amongst virtually all organs in both normal and immunocompromised hosts (Pillay *et al*, 1993a, D'Arminio Monforte *et al*, 1992).

The wide variety of disease manifestations in immunosuppressed patients including retinitis, hepatitis, pneumonitis, and gastrointestinal disease are detailed in table 1.1. There is some debate regarding the significance of HCMV infection in organ dysfunction. There are two possible hypotheses, either HCMV directly causes immunosuppression leading to disease in the immunocompromised host as a result of replication, or the incidence of HCMV infection rises due to immunosuppression resulting in end organ disease. Distinct cytopathogenicity has been defined in patients suffering from AIDS where gastrointestinal disease and HCMV retinitis are common. The macroscopic damage to the epithelial and smooth muscle cells lining the wall of the intestine, seen as perforations or ulcerations is often associated with extensive viral replication (Aqel *et al*, 1991, Kyriazis *et al*, 1992, Singzer *et al*, 1995). The causative role of HCMV in gastrointestinal disease is illustrated further by the resolution of symptoms following antiviral therapy with ganciclovir (Chachoua *et al*, 1987 Dietrich *et al*, 1993a). HCMV retinitis is a progressive disease often leading to blindness due to severe necrotising damage to the retina as a result of HCMV replication. AIDS patients suffering from HCMV retinitis also experience resolution of symptoms following treatment with ganciclovir which must be maintained to prevent disease progression (Jabs *et al*, 1989, Bowen *et al*, 1996).

Immunocompromised transplant recipients also experience organ dysfunction and graft rejection although it remains controversial whether HCMV is directly responsible. For example, renal transplant recipients seldom experience renal failure due to HCMV infection. However, glomerulopathy characterised as enlargement of the epithelial cells and an accumulation of mononuclear cells in the glomerular capillary, has been associated with HCMV viraemia (Richardson *et al*, 1981). This is in contrast to the pathology of HCMV disease in bone marrow transplant recipients who are prone to pneumonitis after transplantation, and only after engraftment of donor marrow. In these patients, pneumonitis is considered to be an immunopathological response, where HCMV

is presumed to trigger a cytotoxic T-cell mediated immune response to virally encoded antigens on the alveolar cells of the lung (Grundy *et al*, 1987). This is further supported by the fact that AIDS patients who often have HCMV in their lungs rarely suffer from pneumonitis, presumably due to the fact that they are unable to mount a sufficient immune mediated response. Nevertheless, HIV patients that have recently seroconverted are able to mount an immune response and therefore appear more prone to HCMV pneumonitis (Squire *et al*, 1992). Liver dysfunction and febrile episodes associated with systemic HCMV infection frequently occur following transplantation. In the case of these two disease associations, there appears to be an indirect cytopathogenic mechanism in place which maybe mediated by the host inflammatory immune response. Thus, different pathogenic mechanisms mediated by the immune response lead to impairment of organ function either by direct or indirect HCMV cytopathogenicity.

In order for the virus to establish productive infection within the infected organ, the host immune response must be impaired or evaded to allow viral replication, or may itself cause indirect cytopathogenicity in the infected tissues. The pathogenic mechanisms that lead to HCMV disease manifestations in the immunocompromised host remain to be fully elucidated, and is further confounded by the lack of animal models available. However, exploitation of the *in vitro* cell tropism of HCMV has led to postulated mechanisms of pathogenesis. HCMV is hematogenously distributed amongst a wide variety of organs and it is proposed that this spread is due to systemic distribution of the virus during primary HCMV infection. The mechanism for reactivation of endogenous virus has not been fully demonstrated, and as with viral latency, *in vitro* studies have primarily been based on the mouse model. In one study, the risk of recurrence was compared in mice infected either during the neonatal period or during adult life (Reddehase *et al*, 1994). In this system, mice that were infected in the neonatal period suffered a higher mortality rate with virus widely disseminated amongst a variety of organs. Conversely, mice infected during adult life had fewer episodes of recurrence and localised virus replication only detected in the lungs. The authors suggest that the burden of latent virus and risk of recurrence were related to the extent of viral replication during primary infection. Resolution of primary infection in a specific organ does not necessarily prevent the establishment of latency at that site (Balthesen *et al*, 1994).

Table 1.1 Major HCMV disease manifestations encountered by the immunocompromised host.

HCMV Disease in the Immunocompromised Host			
Symptoms	Solid Organ Transplantation	Bone Marrow Transplantation	AIDS
Fever/Hepatitis	++	+	+
Gastrointestinal	+	+	+
Retinitis	+	+	++
Pneumonitis	+	++	
Immunosuppression	+		
Myelosuppression		++	
Encephalopathy			+
Polyradiculopathy			+
Addisonian			+
Rejection/GVHD	?	?	

+ <10% patients

++ 11-50% patients

? postulated but not proven association.

It is important to note that animal models utilise viruses that are highly species specific, as are their human counterparts, and as a result this may not reflect the complex course of events encountered by humans. Factors that can influence the course of human CMV disease in the immunocompromised host are primary infection, viraemia, virus load, and the degree of immunosuppression. The identification and quantitation of these risk factors in the immunocompromised host will lead to the components to elucidation of the putative mechanisms that control HCMV disease progression.

1.5 HCMV Latency

Following primary infection, HCMV establishes viral latency at many sites in the infected host, although it is not clearly defined which cells or tissues harbour latent virus. One explanation for this is that HCMV can latently infect the peripheral blood cells which in turn distribute the virus to many organs. The definition of viral latency within the host is also still a matter for discussion, since it has not yet been established whether the virus exists in a truly latent state or exhibits very low level productive infection. Questions that remain to be answered in the context of viral latency are 1) how infected cells escape the host immune response, 2) which are the target cells for viral persistence and 3) what events lead to reactivation of the virus.

One theory is that viral latency is achieved by limited gene expression in non-permissive cells. This is based on *in vitro* evidence, where certain cells only express IE genes following infection with HCMV but upon differentiation, become fully permissive to complete viral replication (Ibanez *et al*, 1991, Sinclair *et al*, 1992, Lathey *et al*, 1991, Poland *et al*, 1994, Lazzarato *et al*, 1994). Monocytes in the peripheral blood have been shown *in vivo* to be a major site of persistence for HCMV using both amplification of viral DNA and hybridisation techniques (Taylor-Wieldman *et al*, 1991, Soderberg *et al*, 1993). *In vivo* studies using monocyte/macrophage cells also show that upon differentiation of monocytes to tissue macrophages, both early and late mRNA transcripts can be detected, indicating that differentiation may be required to support complete viral replication (Singzer *et al*, 1996). Fibroblast cell, in contrast to monocyte derived macrophages, show peak mRNA levels early in the replicative cycle at 5 and 48 hours for early and late gene expression (Fish *et al*, 1995). In addition to this proposed restricted mechanism of gene expression, during late stages of infection, HCMV can be detected in vacuoles derived from the host Golgi network illustrated by the presence of mannosidase II (Fish *et al*, 1996). This sequestering of HCMV within host derived

vacuoles could not only protect the virus from cellular immune responses, but may also serve to prevent damage to the host cell caused by the synthesis of viral proteins. These data taken together suggest that by restricted IE gene expression and vacuole localisation, the virus may be able to escape the host cytotoxic immune system to allow persistence or latency. HCMV can also mediate its own “escape” from the host immune system by expressing a variety of genes as discussed further in section 1.6.3 of this thesis.

1.6 The Host Immune Response to HCMV

1.6.1 The Cellular Immune Response to HCMV

The immune system is undoubtedly vital to control HCMV infection and results in a complex interaction between the virus and the host. HCMV is immunosuppressive and also modulates the immune system by encoding a variety of genes which allow the virus to potentially “escape” immune surveillance. The cellular immune response to HCMV comprises MHC class I restricted cytotoxic lymphocytes (CTL), antibody dependent cytotoxicity (ADCC) and natural killer cells (NK). The primary line of defence for the host against HCMV is the cytotoxic cellular immune response, which in immunocompetent individuals, is sufficient to resolve primary infection but unable to prevent the establishment of latency. This is illustrated by episodes of reactivation observed in healthy seropositive individuals as viral shedding from various body sites following primary infection.

Several targets have been identified that elicit a cytotoxic cellular immune response, including both structural (pp65, pp150, gB and gH) and non-structural proteins (IE), synthesised at early and late times during the replication cycle of the virus. Earlier studies identified CTL responses to IE gene products amongst healthy HCMV seropositive individuals (Borysiewicz *et al*, 1988, Alp *et al*, 1991, Wills *et al*, 1996). However, these initial studies revealed low precursor frequencies of cytotoxic T-cells to the immediate early gene products with limited ability to lyse HCMV infected cells *in vitro* suggesting the importance of other viral antigens to mediate a CTL response (Gilbert *et al*, 1993). Expression of IE gene products was not restricted in human cells, indicating that reduced T-cell killing was due to limited presentation of the protein as opposed to reduced gene expression (del Val *et al*, 1989, Gilbert *et al*, 1993). Indeed, a later study by the same authors described a block in the presentation of IE gene products by selective interaction with the pp65 lower matrix protein (Gilbert *et al*, 1996).

More recent studies have identified the lower matrix protein pp65 (UL83) as the major target for the CTL mediated response during primary infection (McLaughlin-Taylor *et al*, 1994, He *et al*, 1995, Boppana *et al*, 1996a, Wills *et al*, 1996). Further characterisation by fine mapping studies identified three major T-cell epitopes within the C-terminus of pp65 as targets for the CTL response (Khattab *et al*, 1997). The study by Wills *et al* (1996) examined the relative contributions of the CTL responses to IE1, gB and pp65 by measuring precursor frequencies to these antigens in the blood of healthy HCMV seropositive donors. Between 70 and 90% of the CTL response was attributable to pp65 in HCMV infected cells, which was restricted to a single peptide in four out of five donors with the HLA A2 allele, suggesting the possibility of a haplotype restricted response. These data are somewhat surprising, since the α gene products would be the likely candidates for a CTL response due to their early expression in the replication cycle of the virus. However, Gilbert *et al* (1996) discovered that expression of pp65 increased threonine-phosphorylation of IE in vitro, and that this interaction of pp65 with IE gene products did not restrict IE expression but prevented presentation of IE (Gilbert *et al*, 1996). As a result the authors proposed that release of structural viral proteins into the cytosol prior to viral replication, could modify the presentation of endogenously synthesised viral antigens. This block in presentation prior to loading of the peptides without affecting MHC class I presentation in the infected cell, could serve as a mechanism of specific immune escape. Moreover, the immune response elicited to pp65 does not require *de novo* synthesis of the protein since this is the major constituent of the virion tegument which is released after virus entry and prior to viral replication.

Natural killer cells are also important at limiting viral replication prior to the specific induction of the CTL response, however, NK cells alone are insufficient at preventing HCMV disease in the immunocompromised host. The importance of NK cells has been demonstrated using the murine model, where NK cells have been shown to limit HCMV infection upon subsequent lethal challenge (Bukowski *et al*, 1984 & 1985). In a more recent study by Lathbury *et al* (1996) involving mice, the NK response was further characterised to be defined by the genotype of the mouse when using either susceptible, highly resistant or moderately resistant mouse strains (Lathbury *et al*, 1996). This study revealed that in the absence of T-cells, NK cells were responsible for controlling MCMV replication in visceral organs early in infection. In addition, the rat model has demonstrated the importance of NK response to RCMV in both immunocompromised and immunocompetent rats (Van Dam *et al*, 1997). The NK response in immunocompromised rats was markedly increased amongst the visceral organs but not

the salivary gland following infection, with a small increase in CD8+ T-cells with reduced expression of CD8 and TCR. In contrast, immunocompetent rats produced a substantial CD8+ mediated response with limited NK response to control virus replication. Furthermore, the importance of the non-specific immune response has been demonstrated *in vivo* during recovery from HCMV disease in the immunocompromised host (Quinnan *et al*, 1982, Bowden *et al*, 1987, Venema *et al*, 1994). Indeed, Venema *et al* (1994) observed a positive correlation between increased levels of activated NK cells and recovery from HCMV infection in renal transplant recipients. Although the exact role of the non-specific cellular immune response in the immunocompromised host remains to be fully elucidated with larger groups of patients, the ability of NK cells to control HCMV infection should not be underestimated. Indeed, HCMV via the synthesis of the class I homologue, UL18, and down regulation of the MHC class I exhibits a mechanism of NK escape which may play a pivotal role in the ability of the virus to establish latency (discussed further in section 1.6.3).

Given the diversity of strains and the four genotypes of HCMV gB coupled with the variety of proteins that could serve to elicit an immune response, it is unlikely that protective immunity is restricted to one or two antigens. It still remains to be established whether the induction of an immune response directed against specific antigens is sufficient to provide protection against HCMV infection *in vivo*. However, *in vitro* experiments with HCMV have revealed that adoptive transfer of antigen specific CTL clones are sufficient to provide protection from lethal challenge (Reddehase *et al*, 1994). Indeed, the CTL response has been shown to provide protective immunity in bone marrow transplant patients at risk of HCMV pneumonitis (Reusser *et al*, 1991, Riddell *et al*, 1992 & 1994, Walter *et al*, 1995). In these patients, infusion of adoptively transferred CD8+ HCMV specific CTL clones directed against structural proteins limited HCMV viraemia (Walter *et al*, 1995). These data illustrate the central importance of the cellular immune system in controlling HCMV disease, which is further illustrated by the observation that patients with impaired cellular immunity are those most at risk of disease.

1.6.2 The Humoral Immune Response to HCMV

The humoral immune response to HCMV may not play such an important role in the control of HCMV infection in transplant recipients as the previously described cellular immune response. However, there is evidence to suggest that humoral immunity plays a central role in reducing congenital HCMV infection. The importance of antibodies in limiting HCMV infection was first demonstrated in neonates infected postnatally by blood

transfusions. Morbidity and mortality rates were reduced considerably in infants receiving transplacentally acquired anti-HCMV antibodies (de Cates *et al*, 1994). The role of the humoral immune response is clearly illustrated than during pregnancy. It has long been established that transfer of maternal antibodies from HCMV seropositive immune mothers to the fetus can provide protection from subsequent congenital HCMV infection (Stagno *et al*, 1982, Boppana *et al*, 1993, Fowler *et al*, 1992, Donner *et al* 1993, Liptiz *et al* 1997). Furthermore, prophylactic passive administration of pooled and specific immunoglobulin to renal and a lesser extent bone marrow transplant recipients has been shown to reduce the risk of HCMV disease (Snydman *et al*, 1987, Ringden *et al*, 1991). Indeed, administration of T-cell depleted bone marrow from HCMV seropositive donors to seronegative recipients was shown to provide a 50% reduction in the incidence of fatal HCMV disease, suggesting a role for the non-T cell immune responses in mediating protective immunity (Grob *et al*, 1987).

The main targets for the antibody mediated response to HCMV are directed predominantly to the structural envelope proteins gB and gH, both of which elicit neutralising antibodies in the infected host. Indeed, the presence of antibodies to gB correlates directly with neutralising activity and is independent of total HCMV specific IgG titre (Marshall *et al*, 1992). The majority of neutralising antibodies are directed to gB which contains two linear neutralising epitopes AD-1 and AD-2, whereas gH contains only one linear neutralising epitope. Sequence variations within the AD-1 domain of gB leading to coding mutations have been detected amongst renal transplant recipients and patients with AIDS, although the neutralising ability of gB was unaffected (Roy *et al* 1993).

In the mouse model, neutralising antibodies have been shown to reduce episodes of recurrence and to limit virus dissemination amongst the visceral organs (Jonjic *et al*, 1994, Reddehase *et al*, 1994). The importance of neutralising antibodies *in vivo* remains to be clearly defined and there is a distinct lack of studies that have investigated the relationship between neutralising antibodies and HCMV infection in immunocompromised patients. Following primary infection in the immunocompetent host, neutralising antibodies to gB and to a lesser extent gH can be detected in the blood (Rasmussen *et al*, 1991b). In immunocompromised patients neutralising antibodies may be more important at controlling infection due to an impaired T-cell mediated immune response. Indeed, Rasmussen *et al* (1994) compared levels of antibodies to gB and gH in AIDS patients with or without HCMV retinitis. Increased levels of gB antibodies were comparable to HIV seronegative patients with HCMV mononucleosis. The levels of

antibodies to gH while elevated in most HIV seropositive individuals, were lower in patients with CD4 counts below 100cells/mm³. This study did not measure the neutralising ability of either gB or gH, however, there was a correlation between low level antibody response to gH and the risk of HCMV retinitis. Further studies are required to examine the role of neutralising antibodies in HCMV pathogenesis amongst immunocompromised patients.

1.6.3 HCMV and Immune Escape

In immunocompromised individuals it is assumed that the equilibrium between the immune system and the virus becomes disrupted to allow the development of HCMV disease, whereas in the normal host the immune system is sufficient to control infection. HCMV has adapted to the host by acquiring a variety of genes which allow the virus to avoid immune surveillance thus assisting the establishment of viral latency. The genes responsible for immune evasion include UL18 the MHC class I homologue, pp65 the lower matrix protein, US2, US3, US6 and US11. These genes prevent presentation of peptides to the host immune response in the context of MHC class I by different mechanisms, thus avoiding attack by natural killer cells and cytotoxic T-cells as shown in figure 1.5. Studies utilising MCMV revealed that deletion of m144, the analogous gene to UL18 of HCMV, resulted in severely restricted viral replication when compared to wildtype virus (Farrell *et al*, 1997). Attenuation of this phenotype was attributable to attack by natural killer cells revealed by *in vivo* depletion studies. The authors proposed that the m144 gene mimics MHC class I and inhibits NK lysis thereby avoiding early clearance of the virus by the host. Similarly, it has been proposed that the class I homologue gene of HCMV, UL18, allows the virus to avoid attack by natural killer cells, mimicking the function of cellular class I and acting as an NK decoy (Farrell *et al*, 1997, Reyburn *et al*, 1997).

US3 an immediate early gene which encodes an endoplasmic reticulum (ER) resident glycoprotein, prevents intracellular transport of MHC class I molecules to avoid attack by CD8+ T-cells. The US3 gene interacts with the MHC class I molecules during the early stages of virus replication, and retains stable class I heterodimers which are loaded with peptides in the ER (Ahn *et al*, 1996). In contrast, the US11 gene of HCMV which also encodes an ER resident transmembrane type I glycoprotein is synthesised at early times during infection. US11 down regulates the presentation of class I molecules by dislocating newly synthesised class I molecules from the ER and transporting them to the cytosol for rapid degradation by proteosomes (Wiertz *et al*, 1996). Similarly, the US2 gene

product selectively down regulates class I using the same mechanism as US11. US2 binds to newly synthesised class I molecules and transports them from the ER back to the cytosol for deglycosylation and subsequent degradation by resident proteosomes (Wiertz *et al*, 1996). The breakdown intermediate involved is associated with the Sec61 complex and the proteosome itself. The mechanism involved appears to be a reversal of the translocation process in association with the Sec61 complex which forms a protein channel that originally transports the class I molecules into the ER.

The US6 gene expressed at early/late times during infection encodes a 22-kDa glycoprotein that binds directly to the transporter associated with antigen processing (TAP)/class I complex. This mechanism of down regulation of class I is analogous to that of the ICP47 gene of HSV. Peptides are retained in the ER by inhibition of translocation from the cytosol into the ER lumen. Thus, TAP-dependent peptide loading is prevented resulting in down regulation of class I molecules at the cell surface. This block in presentation is abrogated by interferon- γ treatment of US6 transfected cell lines which restores class I presentation comparable to wildtype levels (Lehener *et al*, 1997). In addition, pp65 the abundant lower matrix protein of HCMV selectively prevents class I presentation of the principal immediate early protein which is expressed prior to the blockade in class I presentation (Gilbert *et al*, 1996). These studies reveal the diversity of genes and the complex mechanisms of interaction of HCMV with the host immune system that enable the virus to escape from immune surveillance. It is also interesting to note that the different mechanisms involved appear at different times in the cycle of virus replication. Sequential expression of these genes is involved in immune escape, presumably to maximises the potential of the virus to avoid immune surveillance in order to establish latency or persistent infection within the host.

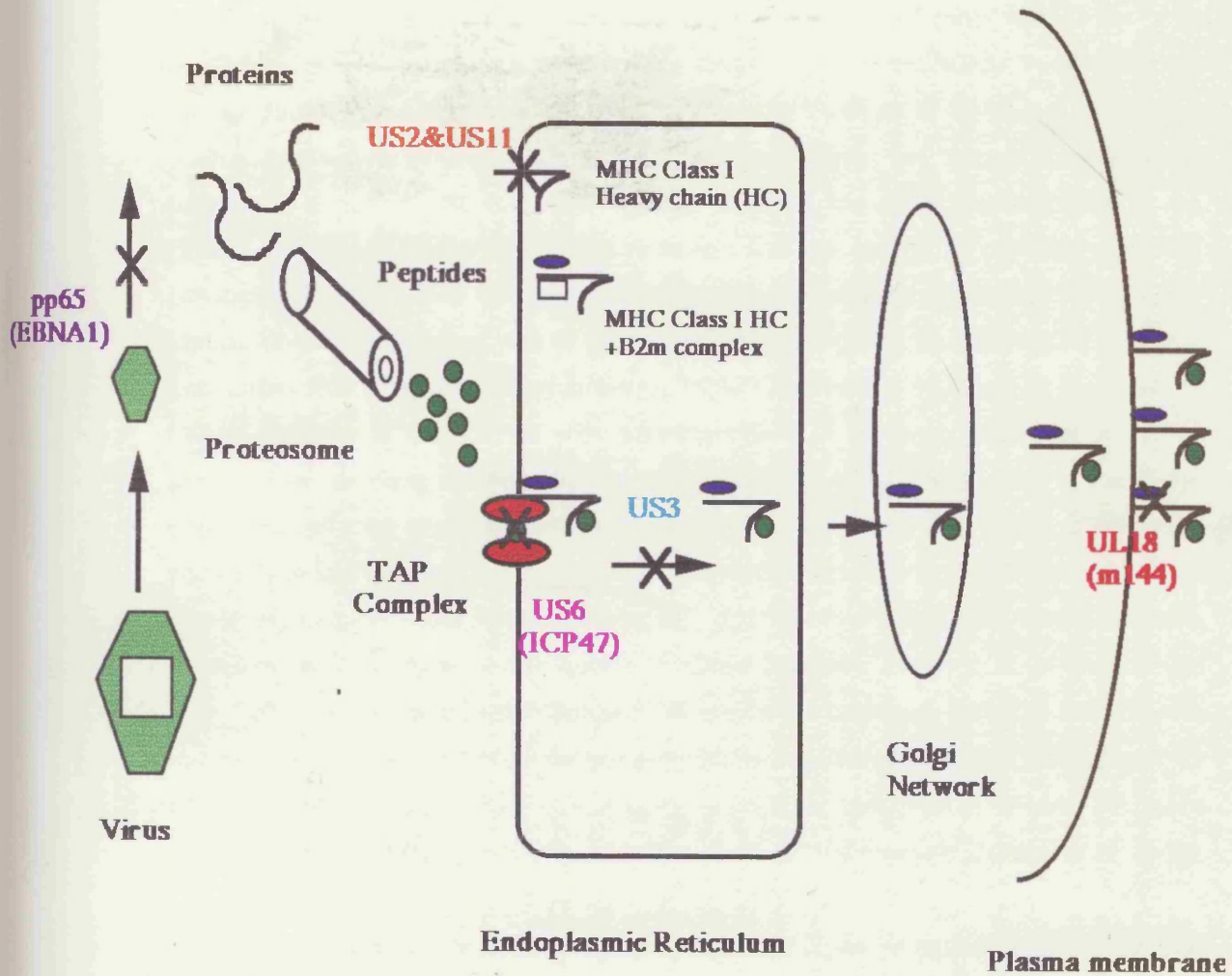


Figure 1.5 Schematic representation of HCMV genes and mechanisms involved in immune escape where analogous genes for other herpesviruses are shown in brackets m144 (MCMV), ICP47 (HSV), EBNA1 (EBV). TAP (transporter associated with antigen processing), β_2m (beta-2 microglobulin), EBNA (Epstein Barr virus nuclear antigen).

1.7 HCMV Infection in the Immunocompromised Host

1.7.1 HCMV Infection in Solid Organ Transplant Recipients

HCMV continues to be an important pathogen following solid organ transplantation, causing disease in the infected host as a result of primary infection, reinfection or by reactivation of endogenous latent virus. The absence of specific HCMV T-cell mediated immunity leads to the progression of disease. The establishment of disease is dependent upon several risk factors including the level of immunosuppression, source of the allograft (either live donor or cadaveric) and HLA mismatching, but relies predominantly on the HCMV serostatus of the donor and recipient and the receipt of unscreened blood products. Hence, HCMV seronegative recipients receiving donor organs from HCMV seropositive donors are at risk of primary infection following transplantation which is associated with a high incidence of severe HCMV disease. A significantly lower risk of HCMV disease is associated with transplantation of a seropositive donor into a seropositive recipient (D+R+). This reduced incidence of HCMV disease in the D+R+ population of solid organ transplant recipients is presumably due to the pre-existing immunity in the recipient. Molecular and seroepidemiological studies indicate that the donor organ itself is the source of infection, and that reinfection with donor strains is associated with an increased incidence of HCMV disease (Grundy *et al*, 1988, Bowden *et al*, 1991). HLA mismatching amongst renal transplant recipients has also been shown to increase the risk of HCMV infection particularly in patients with DR7, DRW6 and B7 HLA types raising the possibility of HLA typing to aid in the reduction of disease incidence when considered in the light of other risk factors for HCMV disease (Boland *et al*, 1993).

The range of disease manifestations observed in solid organ transplant recipients (listed in table 1.1) vary according to the type of transplantation and are presumably related to the level of immunosuppression encountered by the host. Symptoms appear typically within the first three months post-transplantation. The type of HCMV disease encountered ranges from severe end organ involvement (e.g encephalitis, retinitis, adrenalitis, enteritis and hepatitis) to the less severe fever and mononucleosis type syndrome. However, there are common clinical syndromes in the absence of organ involvement associated with HCMV disease in all types of transplant recipients namely fever, leukopenia, thrombocytopenia and malaise.

There is still discussion as to the direct involvement of HCMV in organ dysfunction, although there is evidence to suggest that graft rejection is associated with HCMV infection (Fernando *et al*, 1994, Lautenschlager *et al*, 1995, Stenhoff *et al*, 1996). As

discussed in section 1.4, renal transplant recipients can suffer from a distinct glomerulopathy associated with HCMV viraemia, which dramatically reduces graft survival. In addition, following liver transplantation, vanishing bile duct syndrome, a distinct syndrome of bile duct sclerosis, has also been associated with active HCMV infection during the post-transplant period leading to graft rejection (Martelius *et al*, 1997). The mechanism of graft rejection is not clearly defined although HCMV infection has consistently been associated with poor graft survival. Postulated mechanisms include the increased expression of MHC antigens in the organ itself and so called "molecular mimicry" due to the expression of HCMV encoded MHC I homologues.

Cardiac transplant recipients who are prone to atherosclerosis, provide further evidence of the involvement of HCMV in graft loss. Indeed, HCMV has been consistently associated with accelerated atherosclerosis in the absence of other important variables, which leads to limited long term survival in cardiac allograft transplant recipients. Furthermore, recent evidence has shown that HCMV IE interacts directly with p53 tumour suppressor gene within the vascular epithelium of the heart, resulting in infiltration of smooth muscle cells leading to occlusion of arteriole walls by the development of atherosclerotic plaques (Marx *et al*, 1994, Speir *et al*, 1994). As discussed, the risk of disease is greatly reduced by avoiding transplantation of seropositive donors into seronegative recipients, however, this is not always a practical solution owing to the short supply of organ donors. As a result, considerable emphasis is now placed on reducing the incidence of HCMV disease by identifying those patients most at risk of disease for treatment with effective antiviral therapy.

1.7.2 HCMV Infection in Bone Marrow Transplant Recipients

The HCMV disease associations of bone marrow transplant (BMT) recipients differ markedly to those encountered by solid organ transplant recipients. The disease incidence for allogeneic transplant recipients is between 40-50% (Meyers *et al*, 1986). However, unlike solid organ transplant recipients, the greatest risk of HCMV disease is associated with transplantation of a seronegative donor into a seropositive recipient. Thus, it is the reactivation of endogenous HCMV rather than reinfection of exogenous virus that leads to HCMV disease progression (Winston *et al*, 1985). This suggests that the donor marrow does not present high risk for transmission of HCMV infection to the recipient. On the contrary, the donor marrow may provide cellular and humoral immunity to the recipient, which would account for the lower incidence of HCMV disease observed in patients receiving marrow from HCMV seropositive donors (Grob *et al*, 1987). Indeed,

seropositive donors improve the ability of the recipient to reconstitute their CMV specific cellular immunity following immunosuppression as a result of transplantation.

The incidence of transfusion associated HCMV infection has been reduced to almost zero in bone marrow transplant recipients due to transfusion of HCMV seronegative blood products into seronegative recipients (Bowden *et al*, 1991). Indeed, several studies have shown that the removal of the leukocyte fraction from transfused blood greatly reduces the incidence of transfusion acquired HCMV infection amongst allograft recipients (Bowden *et al*, 1991, Miller *et al*, 1991). The removal of HCMV DNA from blood by filtration has been suggested as an alternative to the use of HCMV seronegative blood by several authors, to reduce the demand on blood supplies (Smith *et al*, 1993, van Prooijen *et al* 1994, Bowden *et al*, 1995). In the most recent of these studies, Bowden *et al* (1995) found no difference in the incidence of HCMV disease between the 502 patients randomised to receive either filtered or seronegative blood products.

There is a significantly higher mortality rate associated with HCMV disease in allogenic BMT recipients, where the predominant disease manifestation is HCMV pneumonitis with an incidence of approximately 10-15%. Prior to the introduction of prophylactic ganciclovir therapy for HCMV infection during the 1990's, disease onset occurred typically within the first two months following transplantation. This has now been shifted to much later times following cessation of prophylactic treatment (Goodrich *et al*, 1993). HCMV pneumonitis in the bone marrow setting is frequently associated with the immunopathological condition, graft versus host disease (GVHD, Meyers *et al*, 1986). However, the role of HCMV in the induction of GVHD is still the topic of much discussion with two main hypothesis. First, the pathogenesis of HCMV pneumonitis is thought to result from uncontrolled viral replication in the lung due to the absence of a HCMV specific CTL response resulting in disease (Reusser *et al* , 1991 Greenberg *et al* , 1991). Alternatively, the second mechanism is thought to be an immunopathological reaction whereby the host immune system leads to pathology in the lung which maybe triggered by HCMV infection (Grundty *et al* , 1988, Jacobson *et al* , 1990).

1.7.3 HCMV Infection in AIDS patients

Human Cytomegalovirus is the commonest viral opportunistic pathogen associated with acquired immunodeficiency deficiency syndrome (AIDS), and has been classified as an AIDS defining illness according to the CDC (Centers for Disease Control, Atlanta U.S.A). Indeed, HCMV seropositivity has been associated with an increased risk for AIDS,

suggesting HCMV as a cofactor in the progression of HIV infection (Webster *et al* , 1989, 1991, 1992). Epidemiological studies have revealed that patients infected with both viruses were more likely to progress faster to AIDS (Webster *et al* , 1991, Detels *et al*, 1994). Several *in vitro* studies have shown that HCMV upregulates HIV replication, however, the implications of these results in humans remain to be defined (Barry *et al* , 1990, Rando *et al* , 1990, Paya *et al* , 1991, Peterson *et al*, 1992). A study by von Laer *et al* (1995) revealed that mRNA from the IE gene could be detected in CD4+, CD8+ and CD19+ lymphocytes of HIV patients with active HCMV infection (von Laer *et al* , 1995). There is *in vitro* evidence to suggest that the selective induction of the expression of Fcγ receptors on the cell surface by HCMV can alter HIV cell tropism allowing HIV replication in cells not normally susceptible to HIV replication (McKeating *et al* , 1990).

The advent of moderately effective antiretroviral therapy led to improved survival of HIV patients with more profound immunosuppression, resulting in an overall increase in the incidence of HCMV disease. In contrast to a decrease in other opportunistic infections common in AIDS patients such as *Pneumocystis carinii* and *Toxoplasmosis* (Hoover *et al* , 1993) . Indeed, the incidence of HCMV infection is much higher in patients with a low CD4+ cell count (Pertel *et al* , 1992, Munoz *et al* , 1993). Patients with a CD4+ count of <50 cells/ μ l have a 20% risk of developing HCMV disease compared to 10% in patients with a CD4+ count of <100 cells/ μ l (Pertel *et al* , 1992, Gallant *et al* , 1992). It remains to be clarified whether the increased incidence of HCMV disease is a result of HCMV accelerating HIV mediated immune suppression or merely reflects HIV directed immune dysfunction to permit increased incidence of HCMV disease.

The most profound HCMV disease observed in AIDS patients affects the central nervous system (CNS) and the GI tract, where HCMV infection results in severe end organ damage (see table 1.1). HCMV retinitis the most common complication associated with the CNS, often leading to blindness due to necrotising damage to the retina. Post-mortem studies revealed up to 90% of patients with AIDS had active HCMV infection, 40% of whom had developed retinitis (Gallant *et al* , 1992). HCMV retinitis was initially thought to result from haematogenous spread to the retinal endothelium via damage to the capillaries during primary systemic infection . More recent *in vitro* studies have revealed that the HCMV receptors reside in the apical surface of retinal pigment epithelial cells (RPE) allowing the virus to spread in a polarised fashion from cell to cell via the lateral membranes (Tugizov *et al* , 1996).

HCMV encephalitis, also observed in patients with AIDS, results in delirium and rapid progression to death and is often confused with HIV related dementia. The pathology of HCMV encephalitis can manifest itself as either subacute or chronic involving the cortex of the brain, or necrotising ventriculoencephalitis resulting in necrotising damage to the cranial nerves (Kalayjian *et al*, 1993). Gastrointestinal disease, the second most profound HCMV associated end organ disease encountered in patients with AIDS, can lead to ulcerations, or rarely, perforations of the epithelial lining due to extensive HCMV replication. Although less common, the damage to the gastrointestinal tract is a significant complication, symptoms include eosophagitis, gastritis, and enterocolitis and also what has often been described as wasting syndrome associated with AIDS (Kotler *et al*, 1991, Dieterich *et al*, 1996). To a lesser extent HCMV disease also affects the peripheral nervous system causing distinct polyradiculopathy as a result of HCMV infection in the spinal cord. Patients often experience weakness of the legs and numbness progressing to flaccid paraparesis and bladder dysfunction (Griffiths *et al*, 1997). Diseases of the peripheral CNS and HCMV associated encephalitis, unlike GI disease and retinitis, respond poorly to antiviral monotherapy with either ganciclovir or foscarnet (de Gans *et al* 1990).

The advent of effective new combination treatment regimens to control HIV infection, (commonly known as highly active antiretroviral therapy or HAART), consisting of treatment with nucleoside analogues plus protease inhibitors, has led to considerably improved immune functions (Collier *et al*, 1996, Deeks *et al*, 1997). This is observed as a reduction in HIV viral load and a marked increase in CD4+ count. Although preliminary, there are data to suggest a reduction in the overall incidence of HCMV retinitis in patients treated with HAART without ganciclovir therapy for HCMV retinitis (Reed *et al*, 1997, Scott *et al*, 1997). This phenomenon presumably reflects an overall reconstitution of the immune system able to control HIV replication and as a consequence HCMV retinitis. However, one report showed no reduction in the incidence of HCMV retinitis despite an increase in CD4+ cells and reduction in HIV viral load, although a delay in the onset of retinitis in the five patients receiving HAART was observed (Jacobson *et al*, 1997). These data suggest that CD4 count alone may not be sufficient to define the immune mechanisms able to reduce HCMV related disease. Indeed, if the majority of the reconstituted CD4+ cells do not recognise HCMV they may not be able to limit HCMV replication. Further controlled trials are required in patients receiving HAART without specific antiviral therapy for HCMV with increasing CD4+ in order to assess the effects of stopping anti-HCMV therapy.

1.7.4 HCMV Congenital Infection

Congenital HCMV infection can manifest itself as a result of damage to the CNS, and has been compared to CNS disease associated with HCMV infection in AIDS patients. As described in section 1.3, congenital HCMV can be acquired either during pregnancy via intrauterine transmission of the virus or perinatally during birth or breast feeding. The latter of the two modes of transmission is associated with less severe sequelae than acquisition of HCMV (Fowler *et al* 1992). Several risk factors have been identified that predispose the infant to severe sequelae including; mode of transmission, the serostatus of the mother, and the gestational age at the time of transmission.

Primary HCMV infection during pregnancy can result in severe neurological sequelae due to fetal damage which is undoubtedly related to the epidemiology of maternal infection. Indeed, there is evidence to suggest reduced vertical HCMV transmission in seropositive mothers, presumably due to the transfer of pre-existing immunity, where women with pre-conceptional immunity rarely if ever deliver children with HCMV infection (Ahlfors *et al* , 1981, Stagno *et al* , 1982 & 1986, Fowler *et al* , 1992). Approximately 8 -10% of babies born to HCMV seronegative mothers have clinically apparent infection, 80 - 90% of whom will develop severe sequelae compared to only 8-15% of asymptomatic babies. The levels of HCMV specific neutralising antibodies are higher in women that transmit virus to the fetus during pregnancy (Alford *et al* , 1986, Bopanna *et al* , 1995 & 1996b). This increase in antibody levels presumably reflects the level of virus replication, where transmission occurs more frequently in women with increased levels of virus replication. This is further supported by the guinea pig model, where increased rates of CMV transmission to the pups is associated with extended episodes of viraemia (Griffith *et al* , 1991).

The range of clinical and laboratory findings associated with symptomatic congenital HCMV infection are shown in table 1.2. Clinical manifestations range from more severe microcephaly which can result in mental retardation, to less severe damage to the visceral organs which is often self limiting. Approximately 80% of babies born with symptomatic congenital HCMV infection will suffer from lasting neurological abnormalities. By far the most common sequela associated with neurological damage in congenital HCMV infection is progressive hearing loss. There is a high rate (between 5-15%) of infants asymptomatic at birth that suffer progressive hearing loss. The incidence of chorioretinitis, another neurological association of congenital HCMV infection, is

Table 1.2 Clinical and laboratory findings associated with symptomatic congenital HCMV infection at birth (taken from Stagno *et al*, 1980)

Clinical/Laboratory Findings	Babies with Abnormality (%)
<i>Clinical:</i>	
Small for Gestational age	50
Petechiae	76
Jaundice	67
Hepatosplenomegaly	60
Microcephaly	53
Lethargy/Hypotonia	27
Seizures	7
<i>Laboratory:</i>	
Hepatocellular damage (ALT>80u/L)	83
Thrombocytopenia (<100x10 ³ /mm ³)	77
Conjugated hyperbiliruinemia (>2mg/dl)	81
Increased CSF protein (>120mg/dl)	46

ALT, alanine transaminase; CSF, cerebrospinal fluid

considerably lower with approximately 12% of symptomatic babies affected. Preliminary data suggest that congenital HCMV infection associated with the CNS involvement can respond to intervention with antiviral therapy (Whitley *et al* , 1997). However, further studies with long term follow up are required to assess the long term potential toxicity of ganciclovir therapy in infants.

The pathogenesis of congenital HCMV infection remains to be fully elucidated, however, several risk factors based on *in vitro* evidence, have been suggested to contribute to disease pathogenesis. These include the quantity of the viral inoculum, the gestational age at the time of virus acquisition and the virulence associated with particular strains of the virus (Stagno *et al*, 1975). Studies using the guinea pig model have shown that increased viral load leads to a poorer prognosis for the offspring (Harrison *et al* , 1995). MCMV infection of severe combined immune deficiency (SCID) mice has revealed different *in vivo* behaviour from different viral isolates, indicating possible differential strain dependent virulence (Reynolds *et al*, 1993). In contrast, the importance of gestational age to the pathogenesis congenital HCMV infection has been based on observational epidemiological evidence. Acquisition of the virus within the first trimester of pregnancy was a significant risk factor for congenital infection although maternal infection in the third trimester also led to fetal damage albeit at a lower frequency (Pass *et al* , 1996).

1.8 Laboratory Diagnosis of HCMV infection

1.8.1 General considerations

Diagnosis of HCMV infection relies on either specific detection of the virus or detection of immunological responses to HCMV. Traditionally, the diagnosis of HCMV related disease or infection relied upon the detection of serum IgG antibodies to the virus. With the advent of new diseases associated with HCMV infection in a variety of patient populations, and the need to implement antiviral treatment regimens to control infection, diagnostic virology for HCMV has changed dramatically. Indeed, as will be discussed later in this chapter, cell culture and virus isolation techniques are becoming rapidly superseded by more rapid modern molecular biological techniques such as DNA /RNA PCR methodology and *in situ* hybridisation techniques. The ability of PCR based technology to provide both diagnostic and prognostic information will be discussed later in section 1.8.5. For the purpose of this thesis a limited overview of the diagnostic virology employed to detect HCMV infection in the immunocompromised and

immunocompetent host will be provided.

1.8.2 Serology

The detection of serum IgG antibodies to HCMV is still important for the identification of previous exposure to HCMV infection, particularly in transplant recipients, HIV infected individuals or pregnant women at risk of transmitting HCMV to the fetus. Serum samples from prospective donors and recipients are routinely screened for the presence of HCMV specific IgG antibodies prior to transplantation as are pregnant women with suspected primary HCMV infection. Although the detection of HCMV IgG is indicative of previous exposure to the virus, it is important to note that the presence of HCMV IgG is not a marker for protection against either reactivation of endogenous or activation of exogenous virus. A variety of formats are available for the detection of serum IgG including; immune haemagglutination, latex agglutination, immunofluorescence, virus neutralisation, radioimmunoassays (RIA's) and enzyme linked immunoabsorbant assays (ELISA's). The respective sensitivities and specificities of these assays for the detection of HCMV IgG are listed in table 1.3. ELISA based assays are the most commonly used in many laboratories due to increased sensitivity and rapidity over existing in house assays. The source of substrate has recently changed to the use of recombinant derived proteins as the source of antigen due to the variability of lysates obtained from virus infected cells (Landinni *et al* , 1990&1995, Kropff *et al* , 1993).

Detection of HCMV specific IgM for the identification of primary infection is questionable due to variability in sensitivity of the assay. This variability is observed since not all patients undergoing a primary infection produce an HCMV specific IgM response. Therefore serology for IgM is usually restricted to immunocompetent individuals with suspected recent exposure to the virus presenting as mononucleosis type syndrome. The most consistent method is radioimmunoassay which detects serum IgM in a variety of patient groups (Griffiths *et al* , 1980, 1982a, 1982b, Rasmussen *et al* , 1982). However, more recently these assays have been replaced with the ELISA format due to concern over the use of radioactivity. Unfortunately this has led to a loss in sensitivity of between 50-90% in immunocompetent individuals with acute HCMV infection (Lazzarotto *et al* , 1992).

Table 1.3 Performance characteristics of several assays for the detection of HCMV specific IgG in human sera (from Principles and Practice of Clinical Virology, Third edition)

Performance Characteristics				
Assay	Specificity	Sensitivity	Objectivity	Rapidity
Neutralisation	++	+	+	-
CF	+	+	+	+
IFA-LA	-	++	-	++
ACIF	+	++	+	++
Latex agglutination	++	++	+	+++
RIA	+	+++	++	++
EIA	+	+++	++	++

ACIF= anticomplement immunofluorescence

CF= complement fixing

EIA= enzyme immunoassay

IFA-LA= immunofluorescence assay for viral late antigens

RIA= radioimmunoassay

1.8.3 Cell Culture

The isolation of HCMV from clinical specimens in fully permissive fibroblast cells, derived from either human foreskin fibroblast (HFF) or human embryo lung (HEL) cells is the standard upon which all other assays for the diagnosis of HCMV are compared. Clinical specimens are inoculated onto semi-confluent monolayers of cells which are carefully monitored until the appearance of characteristic cytopathic effect (cpe) or for a period of up to 28 days due to the slow growth of the virus. An example of the characteristic cpe observed in HEL cells is shown in figure 1.6. A variety of clinical specimens can be used as viral inoculum including saliva, blood, urine and tissue homogenates. The time taken for the appearance of cpe led to modification of conventional cell culture to enhance the growth of the virus in this system. Modifications to the assay include centrifugation and the detection of immediate early antigens in clinical specimens by immunofluorescence. However, conventional cell culture is still the method of choice for diagnosing congenital HCMV infection where cpe can appear as soon as 48 hours in certain cases dependent upon the quantity of virus in the inoculum. For transplant recipients, isolation of HCMV from blood provides the greatest predictive value for HCMV disease (Meyers *et al* , 1990, Ljungman *et al* , 1990 & 1993, Kidd *et al* , 1993).

1.8.3.1 The shell vial assay and detection of early antigen fluorescent foci (DEAFF)

The shell vial assay and the DEAFF test are modifications of conventional cell culture techniques which rely on the detection of immediate early antigens in clinical specimens using monoclonal antibodies. These techniques have replaced the use of virus isolation in many laboratories since results from either assay can be obtained within 48 hours with comparable sensitivity and specificity to conventional cell culture. The shell vial assay (named according to the container used), is the preferred method for the U.S.A which has a sensitivity of between 70-90% and a specificity of almost 100% (Gleaves *et al* , 1984). Unfortunately not all clinical specimens are appropriate for this assay, indeed blood cultures show greater sensitivity in virus isolation. The DEAFF test, the preferential method of choice for the U.K, has a sensitivity of 78% compared to 76% for virus isolation (Pillay *et al* , 1992).

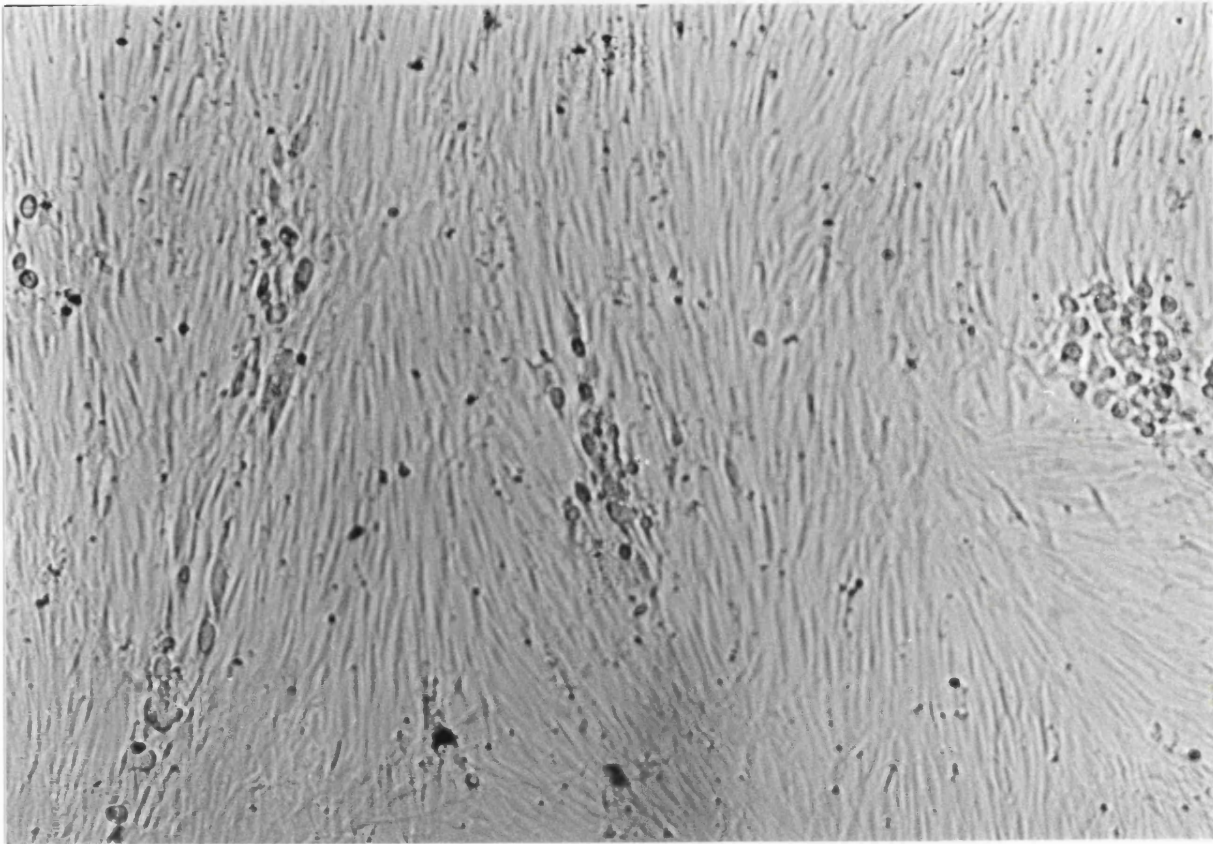


Figure 1.6 Photograph of human embryo lung fibroblasts showing the focal cytopathic effect typical of HCMV (Kindly provided by Mr. Jeremy Bishop).

1.8.4 Antigenaemia

Monoclonal antibodies can be used for the direct detection of antigens in polymorphonuclear leukocytes (PMNL's). The lower matrix protein pp65 is the most abundant antigen detected in circulating leukocytes of HCMV infected individuals and is now known to be the target for the antigenaemia assay (Gerna *et al* , 1991, Grefte *et al*, 1994). Antigenaemia is a semiquantitative assay relying on the detection of pp65 antibody reactive polypeptides in polymorphonuclear leukocytes (PMNL's) circulating in the blood of HCMV infected individuals. Demonstration of immunofluorescence in the nuclei of PMNL cells indicates a positive result. The number of antigen stained cells can be counted relative to the number of known input of PMNL's allowing a semi-quantitative estimate of HCMV viral burden. Results can be obtained within 5 hours of processing of the sample with comparable sensitivity to conventional cell culture. However, samples need to be processed almost immediately and at least 10^5 cells must be counted. In addition, there is concern over the source of pp65 virion associated antigen as a marker for HCMV infection, since the detection of pp65 does not require any prior DNA replication and therefore may not be truly representative of systemic viraemia due to HCMV replication (Grefte *et al* 1994). False negative results may also be obtained in patients experiencing neutropenia since antigenaemia relies on the presence of polymorphonuclear cells.

Nevertheless, antigenaemia has been shown prospectively in a variety of immunocompromised transplant recipients to be indicative of HCMV infection (van der-Bij *et al* , 1988, Schmidt *et al* , 1995, Barber *et al* , 1996). For example in the renal transplant population the presence of 10 positive cells in 50,000 leukocytes represents significant antigenaemia (van den Berg *et al* , 1989). In more severely immunocompromised patients such as allogeneic bone marrow transplant recipients, any level of antigenaemia may be significant (Boeckh *et al* , 1992). There have been encouraging reports regarding the utility of antigenaemia for the prediction of invasive HCMV disease in patients with HIV infection (Landry *et al* , 1993, Bek *et al* , 1996, Reynes *et al* , 1996). Bek *et al* (1996) evaluated the antigenaemia assay in 174 HIV infected patients which detected HCMV in 96.7% of patients with HCMV disease. However, the positive predictive value was only 47% with a sensitivity of 91% , which was dependent upon the number of cells analysed. Indeed, in a similar study by Reynes *et al* (1996), the positive predictive value of the assay could be increased from 45% to 93% if >100 positive cells per slide were analysed, although this resulted in a reduction in sensitivity from 94% to 80%. These data suggest that there is a delicate balance between

sensitivity and specificity of the assay and the number of cells counted indicating the requirement for standardisation when utilising this assay for the analysis of multiple samples from different patient populations.

1.8.5 Detection of HCMV DNA

The detection of HCMV DNA in clinical samples was initially based on hybridisation techniques which were extremely labourious with poor sensitivity in comparison to conventional cell culture (Pillay and Griffiths *et al* 1996). The use of immunocytochemistry for DNA hybridisation is useful for the diagnosis of localised HCMV disease such as hepatitis and colitis, but is limited due to the invasive techniques required to obtain samples (Naoumov *et al* ,1988, Barkholt *et al*, 1994). Modern hybridisation techniques such as the Digene hybrid capture system (DHCS) and the Murex hybrid capture CMV DNA system have improved the detection of HCMV DNA in clinical samples (Mazzulli *et al*, 1996, Bossart *et al* , 1997). More recently, the technology has focused on the rapid direct detection of HCMV DNA in samples by polymerase chain reaction (PCR) or *in situ* PCR techniques. The basis of PCR methodology is described in detail in chapter 2 section 2.1. Briefly, specific primers complementary to a highly conserved region of the genome are selected for DNA amplification. Repeated cycles of denaturation, annealing of the primers to the target sequence and elongation of the DNA are performed to yield a PCR product of the predicted fragment length according to the location of the specific primers.

Unfortunately, due to extreme sensitivity, this technique is subject to contamination and relies on the use of expensive reagents. Contamination can be controlled by strict separation of the individual stages in setting up the PCR reaction into designated areas (Kwok *et al* , 1989). The level of sensitivity of the PCR reactions can be set during optimisation to prevent the detection of latent virus genomes, or alternatively nested PCR approaches can be employed for the detection of small quantities of virus present in tissues. *In situ* PCR techniques are useful for the detection of HCMV DNA in paraffin-embedded tissues as well as from biopsy material to indicate HCMV related organ disease (Burgart *et al*, 1991, Shibata *et al* , 1992, Wolff *et al* , 1993, Brainard *et al* , 1994). The ability to process numerous clinical samples in a short time, has led to the application of PCR to a diverse range of clinical settings. Indeed, there are many reports illustrating the utility of PCR for the detection of HCMV in a variety of clinical samples from different immunocompromised patient groups including; HIV infected individuals, renal, liver and bone marrow transplant recipients and congenitally infected infants (Kidd

et al , 1993, Donner *et al* , 1993, Revello *et al* , 1995, Patel *et al* , 1995, Schmidt *et al* , 1995, Bowen *et al* , 1996, Caballero *et al* , 1997, Abecassis *et al* , 1997, Stephan *et al* , 1997). The versatility of PCR allows DNA detection from a variety of clinical samples including saliva, blood, urine, serum, CSF and BAL samples. To control for variability amongst clinical samples, the quantity of extracted DNA or the number of input cells into the PCR reaction can be kept constant. The negative predictive values for the detection of HCMV DNA in clinical samples are usually high ranging between 95-100%. However, in earlier studies the the positive predictive values for PCR detection of HCMV DNA were considerably lower.

The amplification of HCMV DNA from clinical samples has also been shown to provide prognostic information in a variety of immunocompromised hosts (Kidd *et al* , 1993, Donner *et al* , 1993, Bowen *et al* , 1996, Lao *et al* , 1997, Stephan *et al* , 1997). In the latter of these studies by Stephan *et al* (1997) compared PCR detection of HCMV DNA in whole blood and BAL specimens in lung transplant recipients, the positive predictive value was improved from 50% to 67% in BAL samples. Indeed, the choice of clinical sample analysed can greatly effect the ability of the PCR reaction to predict HCMV disease (Patel *et al* , 1995, Schmidt *et al* , 1995, Hebart *et al* , 1996).

The efficacy of HCMV specific antiviral therapy can also be assessed by PCR since the detection of HCMV DNA in blood, often referred to as DNAemia, is indicative of HCMV replication. In some patients receiving antiviral therapy HCMV DNAemia is reduced to undetectable levels in association with resolution of symptoms. More recently the development of PCR assays has focused on quantitative assays for the measurement of HCMV viral load in clinical specimens. Indeed, HCMV load when measured longitudinally in different patient groups has been shown to provide information into the pathogenesis of viral replication, antiviral efficacy and identifies patients with higher viral loads who are more likely to develop HCMV disease (Fox *et al* , 1995, Gerna *et al* , 1994 & 1995, Drouet *et al* , 1995, Boivin *et al* , 1996, Bowen *et al* , 1996, Spector *et al* , 1998). Thus, PCR based technology for the detection of HCMV DNA can be exploited to monitor immunocompromised patients and generate important information which identifies patients at risk of disease allowing intervention with effective antiviral therapy. The use of a fully quantitative PCR assay to assess efficacy of antiviral therapy and to monitor congenitally infected infants, renal and liver transplant recipients is discussed in detail in chapters 3, 4, and 5 and 6 of this thesis.

1.8.6 Detection of HCMV mRNA

The advantage of the detection of HCMV mRNA is the ability to differentiate between active viral replication as opposed to detection of abortive replication which may be detected by PCR for HCMV DNA. Viral mRNA detection in both tissues and blood can identify the course of HCMV infection at the replication level and may be more indicative of the pathogenesis of virus replication and antiviral efficacy. Several reports have utilised reverse transcriptase PCR (RT-PCR) to elucidate the kinetics or a variety of HCMV genes *in vitro* (Gozlan *et al*, 1992, von Lear *et al*, 1995, Kondo *et al*, 1996). Von Lear *et al* (1995) used RT-PCR to identify the cell types able to support complete viral replication by detection of immediate early and late gene transcripts. The results showed that all major leukocyte populations were productively infected indicating a possible role for PMNL's in the dissemination of HCMV during active infection. Kondo *et al* (1996) utilised RT-PCR to identify the expression of viral transcripts during latency in macrophage progenitor cells of HCMV seropositive individuals. This study revealed the differential expression of latency associated transcripts from the IE1/IE2 region of the genome, with atypical IE1 expression during latency. These studies demonstrate the ability of RT-PCR to provide information on the pathogenesis and kinetics of HCMV replication.

With regard to the use of RT-PCR in the clinical setting, several reports have attempted to use qualitative assays to examine the kinetics of viral replication in clinical samples (Bitsch *et al*, 1993, Gozlan *et al*, 1993, Randhawa *et al*, 1994, Zhang *et al*, 1995, Meyer-Konig *et al*, 1995, Nelson *et al*, 1996, Wolff *et al*, 1996, Gozlan *et al*, 1996, Gaeta *et al*, 1997). The choice of target sequence as with DNA PCR affects the sensitivity of the assay with immediate early transcripts being detected more frequently than late gene transcripts (Bitsch *et al*, 1993, Gozlan *et al*, 1993). The specificity is improved considerably if late gene transcripts are used as the target, since late mRNA detection indicates complete viral replication (Nelson *et al*, 1996). In contrast to detection of HCMV DNA, viral RNA becomes undetectable in patients treated with ganciclovir (Wolff *et al*, 1996, Gaete *et al*, 1997). The positive predictive value of RT-PCR is frequently lower than DNA PCR, which may be attributed to lower sensitivity of some assays. More recently, application of nucleic acid-based sequence analysis assay or NASBA, commonly used for the detection and quantitation of HIV and hepatitis C viral RNA has also been applied to HCMV (Blok *et al*, 1998). In this study, the detection of the late pp67 gene of HCMV provided prognostic information in renal transplant recipients with a specificity of 100% and positive predictive value of 100%.

1.9 Immunosuppressive therapy

1.9.1 General considerations

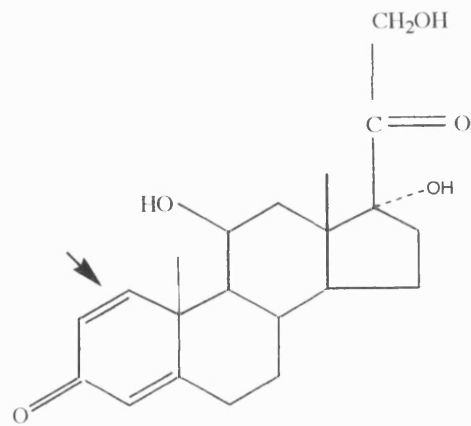
To prevent rejection of the transplanted organ or bone marrow, patients are immunosuppressed with a variety of drugs. The treatment regimens implemented are dependent upon the level of immunosuppression required and the type of transplant, but are generally aimed at suppressing T-cell functions of the host. A combination of immunosuppressive drugs are administered directly after transplantation of the organ into the recipient. In contrast, bone marrow transplant recipients undergo aggressive immunosuppressive therapy prior to infusion of the donor marrow. Bone marrow recipients receive total body irradiation (TBI) usually in combination with chemotherapy to completely eradicate the immune system. Donor marrow is selectively T-cell depleted with monoclonal antibodies prior to re-infusion into the recipient. Solid organ transplant recipients receive a combination of triple immunosuppressive therapy, the doses and combinations of which are modified in the advent of organ rejection and tolerance as measured by renal and hepatic function. The following section describes the main immunosuppressive drugs used for liver and renal transplant recipients relevant to this thesis.

1.9.2 Steroidal Immunosuppressive drugs

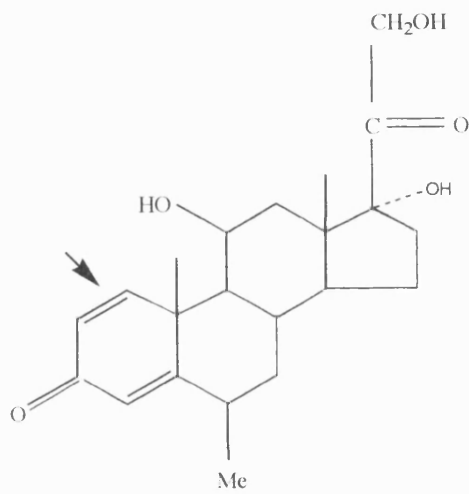
1.9.2.1 Prednisolone and Methylprednisolone

Prednisolone and methylprednisolone are powerful anti-inflammatory agents and members of the glucocorticoid group of steroid hormones which have a characteristic 21-carbon structure shown in figure 1.7. Steroids are metabolised to water soluble glucocorticoids in the liver which are subsequently excreted in the urine. The activity depends on conversion *in vivo* in the liver of the hydroxyl group at carbon-11. Extensive methyl group substitution as for methylprednisolone, slows the rate of metabolism and extends the half life of the drug. Entry into the cells is by passive diffusion or specific mechanisms, while the mechanism of action relies on binding to steroid receptors in the cell cytoplasm. The receptor steroid complex then translocates to the nucleus and interacts with chromatin to modulate gene expression.

a)



b)



c)

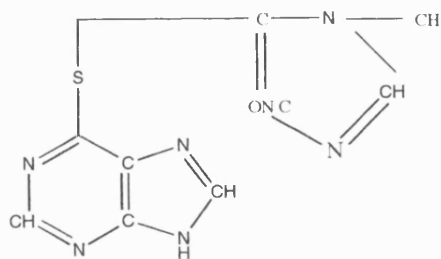


Figure 1.7 Chemical structure of the immunosuppressive drugs a) prednisolone, b) methylprednisolone and c) azathioprine. The arrows indicate the 11-carbon hydroxyl group important for activity of the glucocorticoids.

The effect on the immune system is extensive, with a variety of immune functions being affected. However, the predominant effects in humans are immunoregulatory and directed against cytokine production, cell function and cell traffic. Cytokine synthesis is depressed with down regulation of IL-1, IL-2 and tumour necrosis factor (TNF). Indeed in the presence of steroids, T-cells are unresponsive to IL-1 and unable to synthesise IL-2. Phagocytosis mediated by either neutrophils or monocytes is also impaired. The numbers of circulating leukocytes are reduced dramatically resulting in lymphocytopenia within hours of administration. T-cell populations are affected more than B-cells, and within the subset of T-cells, CD4+ cell numbers are reduced more than CD8+ cells. Differentiation of monocytes to macrophages is blocked as is the ability of monocytes to express the Fc receptor. In contrast, steroids do not alter NK cell function or effectors of the ADCC. When administered as monotherapy, steroids do not appear to be potent immune inhibitors. However, when used in combination with other immunosuppressants they are powerful adjuvants, thus combinations containing steroids are useful to modulate rejection in transplant recipients.

1.9.3 Non-steroidal immunosuppressive drugs

1.9.3.1 Azathioprine

Thiopurine compounds are purine analogues the two most commonly used for immunosuppression are 6-mercaptopurine and azathioprine. The structure of azathioprine is closely related to the structure of hypoxanthine with an imidazole group attached to the sulphur atom at the 6-hydroxyl position as shown in figure 1.7. Specifically, azathioprine is a nitroimidazole derivative of the purine antagonist 6-mercaptopurine, which is rapidly converted in vivo to the parent compound (6-mercaptopurine). Azathioprine is metabolised intracellularly to the active form consisting primarily of thio-inosinic acid by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Azathioprine is a cytotoxic drug which is not selective for lymphocytes alone but acts on dividing cells at the S phase of the cell cycle. Specifically, DNA and to a lesser extent, RNA and protein synthesis are inhibited by thio-inosinic acid due to interference with purine metabolism. For this reason azathioprine is described as a phase specific drug which has broad immunosuppressive activities which can also kill non-lymphoid cells including haematopoietic precursors and proliferating cells.

Primary immune responses are impaired more readily than secondary immune responses, with memory lymphocytes being unaffected. The T-cell population of cells is inhibited to a greater extent than B-cells, while the major effect is to suppress killer and

NK cell activity. Numbers of circulating monocytes and neutrophils are reduced in a dose dependent manner due to cytotoxicity towards haematopoietic precursors. Azathioprine is often used in combination with corticosteroids such as methylprednisolone to suppress the immune system immediately following transplantation. Patients who develop toxic side effects or require maintenance immunosuppression after discontinuing cyclosporin can be successfully treated with azathioprine.

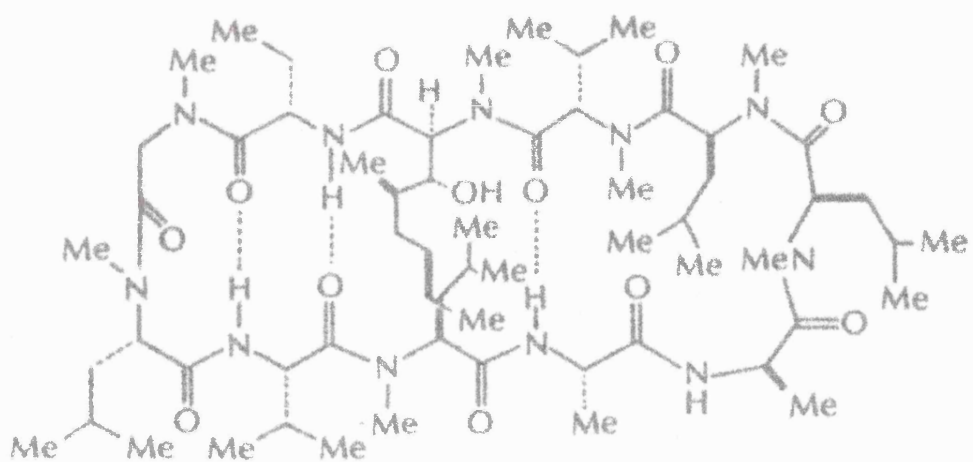
1.9.3.2 Cyclosporin

Cyclosporin is a complex cyclical peptide derived from the fungus *Tolypocladeium inflaticum* consisting of 10 aliphatic amino acids and additional unique amino acid the complex structure of which is shown in figure 1.8. The drug is not water soluble and must be administered with a lipid vehicle either orally or intramuscularly. The mechanism of action is to bind to a class of cytoplasm proteins known as immunophilins which have peptidyl-prolyl isomerase activity. Cyclosporin selectively impairs T-helper cell function by blocking the early phase of developing immune responses, in contrast, suppressor cell functions are unaffected. Specifically, cyclosporin binds to cyclophilin preventing signal transduction of the signal to the nucleus after an intracellular rise in calcium levels. Expression of the early genes activated by T-cell receptor ligation are inhibited including mRNA for the transcription of IL-2 and other cytokines. The synthesis of IL-2 is necessary for the proliferative response of CTL's required for allograft rejection, in contrast the lytic activity of CTL's remains unaffected. Thus, cyclosporin selectively blocks the immune responses involved in graft rejection by inhibiting the activity of T-helper cells. Glucocorticoids and cyclosporin act synergistically when administered simultaneously. Cyclosporin directly inhibits IL-2 while steroids block monocyte/macrophage release of IL-1. Cyclosporin is the primary effective drug used for the treatment of allograft rejection and may be also used for the prevention of GVHD, but is ineffective at treating established GVHD. It is a relatively safe drug and unlike azathioprine is non-toxic to bone marrow stem cells.

1.9.3.3 FK506

FK506 is a macrolide lactone derived from the fungus *Streptomyces tsukubaensis* which is at least 100 times more potent at suppressing transplant rejection than cyclosporin. The mechanism of action is analogous to cyclosporin although both compounds are biochemically distinct the structure is shown in figure 1.8. Both drugs bind to cytoplasmic proteins with peptidyl-prolyl isomerase activity referred to as immunophilins which are

a)



b)

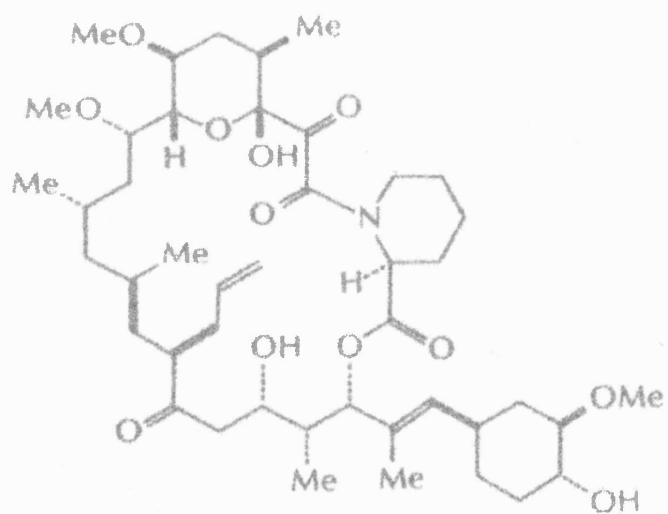


Figure 1.8 Chemical structure of cyclosporin and FK506 immunosuppressants.

believed to be important in transducing signals from the cell surface to the nucleus. As with cyclosporin, T-helper function is primarily affected with significant reduction in IL-2 synthesis and secretion. In contrast, target T-cell killing is unaffected and FK506 is not lympholytic or toxic. There are however, long term complications with continued chronic immunosuppression and increased incidence of opportunistic infections as will be discussed further in chapter 5 and 8 of this thesis.

1.10 Antiviral Therapy

1.10.1 General considerations

There are currently two main antiviral drugs administered for HCMV infection and resulting disease namely aciclovir and the more potent compound ganciclovir. Antiviral therapy can be administered in three ways; either prophylactically prior to onset or detection of systemic infection, pre-emptively prior to any onset of symptoms but following detection of systemic infection, or finally as treatment of established HCMV disease. The most cost effective and ideal administration of antiviral therapy is to treat patients pre-emptively avoiding unnecessary exposure to the toxic side effects of the drugs. However, in the past this was not always possible due to unreliability of the assays for identification of systemic HCMV infection, which were primarily based on cell culture techniques which were also slow to yield results. Thus, patients with systemic infection identified by this method had in many cases already established HCMV disease. Earlier studies examining antiviral efficacy were based on treatment of HCMV disease or prophylaxis. With the advent of more rapid and reliable PCR or antigenaemia based techniques for the detection of systemic infection together with the identification of risk factors for HCMV disease, treatment of HCMV infection is progressing to pre-emptive therapy. In addition to aciclovir and the nucleoside analogue ganciclovir, there are also a number of other antiviral drugs for the treatment of HCMV such as foscarnet which is used as an alternative treatment in the advent of drug resistance to ganciclovir. There are also a variety of new compounds which are at various stages of development. For the purpose of this thesis a brief description of the antivirals used only in the transplant setting follows as relevant to this thesis.

1.10.2 Aciclovir

Aciclovir (known generically as 9-[2-hydroxyethoxymethyl]guanine, with the product name Zovirax) is an acyclic analogue of deoxyguanosine. The structure of the compound is based on guanosine with the ribose sugar moiety replaced by an acyclic side chain and is shown in figure 1.9. Activity of the compound was first discovered against HSV and

VZV. In contrast, activity against HCMV is somewhat variable with many clinical isolates requiring high doses *in vitro* (Biron *et al* , 1980, Datta *et al* , 1980, Shaeffer *et al* 1996). The mechanism of action for HSV and VZV is well defined and is mediated by the thymidine kinase (TK) gene present in both viruses as shown in figure 1.10. The compound is taken up by cells and phosphorylated to the monophosphate form by the virus encoded TK, cellular kinases subsequently phosphorylate the compound to the di and triphosphate form. The active aciclovir triphosphate acts as a competitive inhibitor for the viral DNA polymerase. Aciclovir has a single hydroxyl group on the acyclic sugar moiety corresponding to the 5'-hydroxyl group on the normal sugar of the deoxyguanosine. This feature is important as it is this hydroxyl group that allows linkage of the next incoming nucleoside in the elongating DNA chain by formation of a second phosphate ester bond. Since aciclovir lacks the 3'-hydroxyl group necessary to form this 3'-5' phosphodiester bond aciclovir incorporation into the DNA chain leads to chain termination (Schaeffer *et al* , 1996, Furman *et al* , 1996). Aciclovir can be administered either topically orally or intravenously and is well tolerated *in vivo* following administration with few adverse effects reported. Uninfected cells only display very low levels of aciclovir which may also be a contributing safety feature of the compound. In addition, aciclovir is neither bone marrow toxic nor immunosuppressive (Steele *et al* , 1980, McGuffin *et al* , 1980). Plasma levels are greatest, reaching approximately 10 μ g/ml following a 5mg/kg iv dose compared to only 0.6 μ g/ml after an oral dose of 200mg, thus oral bioavailability is approximately 15-30%. Valaciclovir the valine ester of aciclovir, which is cleaved into aciclovir and valine during a single step catalysed by valine esterase shows increased bioavailability of up to 54% . In addition, famciclovir, the prodrug of penciclovir another nucleoside analogue based on the structure of aciclovir, shows increased bioavailability up to 77% upon cleavage and oxidation to the parent compound. The safety and efficacy of famciclovir against a variety of herpes virus infections is currently under way. Aciclovir is often used for primary prophylaxis and treatment of herpes virus infections particularly HSV in seronegative transplant patients. The effects at reducing HCMV infection are somewhat limited due to the lack of efficacy of this drug against HCMV, which is presumably related to the lack of a virally encoded TK gene. However, prophylaxis in renal transplant recipients has shown to reduce the incidence of HCMV disease (Meyers *et al* , 1988, Balfour *et al* , 1989, Stratta *et al* , 1992).

1.10.3 Ganciclovir

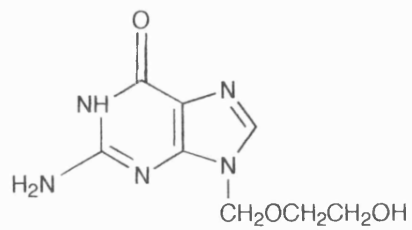
Ganciclovir (known generically as 9-[1,3-dihydroxy-propoxymethyl] guanine with the product name Cytovene) like aciclovir, is an acyclic analogue of deoxyguanosine, the

major difference is that ganciclovir has a hydroxyl methyl group (shown in figure 1.9). This apparently minor difference confers a marked improved antiviral activity against HCMV but also an increase in cellular toxicity. Ganciclovir is effective against a broad range of herpes viruses including HSV 1 and 2, VZV, EBV and HHV-6. The mechanism of action of the compound is the same as for aciclovir and relies on monophosphorylation by the TK gene of HSV and VZV followed by additional phosphorylation to the di and triphosphate forms by cellular enzymes as shown in figure 1.10. HCMV as mentioned previously does not possess a virally encoded TK gene. However, ganciclovir is phosphorylated to the monophosphate form by a virally encoded protein kinase the UL97 gene product (Littler *et al* , 1992, Sullivan *et al* , 1992). Further phosphorylation to the di and triphosphate forms is carried out by cellular kinases, and as with aciclovir, the triphosphate form is a competitive inhibitor of the viral DNA polymerase which when incorporated into the DNA chain slows down elongation. However, unlike aciclovir, ganciclovir is not an obligate chain terminator due to the presence of the 3'hydroxyl group. The lack of this safety feature possessed by aciclovir may explain the increased toxicity of the ganciclovir especially to uninfected cells where the concentration of ganciclovir is considerably higher than that of aciclovir.

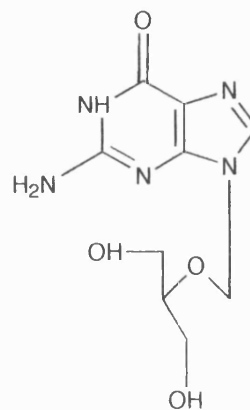
Ganciclovir can be administered either orally or intravenously and recently intraocular implants have been developed for the treatment of HCMV retinitis. Following an iv injection of 5mg/kg dose of ganciclovir, the peak level in plasma reaches approximately 8 μ g/ml. In contrast, as with aciclovir, the oral bioavailability of ganciclovir is poor at between 5-9%.The improved activity against HCMV is partly attributable to the increased intracellular half-life of ganciclovir triphosphate in excess of 18 hours. Two controlled trials have evaluated the efficacy of oral ganciclovir in the prevention of HCMV retinitis in patients with HIV (Brosgart *et al* , 1996, Spector *et al* , 1996). The study by Spector (1996) showed a significant reduction in HCMV isolation in contrast to no reduction in the former study by Brosgart *et al* (1996), highlighting the need for further controlled trials.

The therapeutic use of iv ganciclovir has been established in patients with HIV at risk of HCMV retinitis, where ganciclovir has successfully been used to prevent and treat disease. Following high dose of induction therapy, patients are maintained on low dose ganciclovir, which appears to be the most effective treatment for the prevention of HCMV retinitis (Bowen *et al*, 1996). In solid organ transplant recipients ganciclovir is effective at reducing the incidence of invasive HCMV disease when administered as either primary prophylaxis or more recently as pre-emptive therapy (Schmidt *et al* 1991, Duncan *et al*,

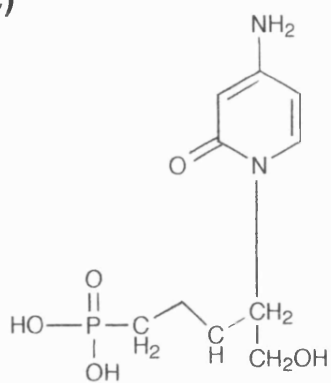
a)



b)



c)



d)

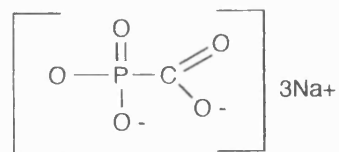


Figure 1.9 Chemical structures a) aciclovir and b) ganciclovir, c) cidofovir and the non-nucleoside d) foscarnet.

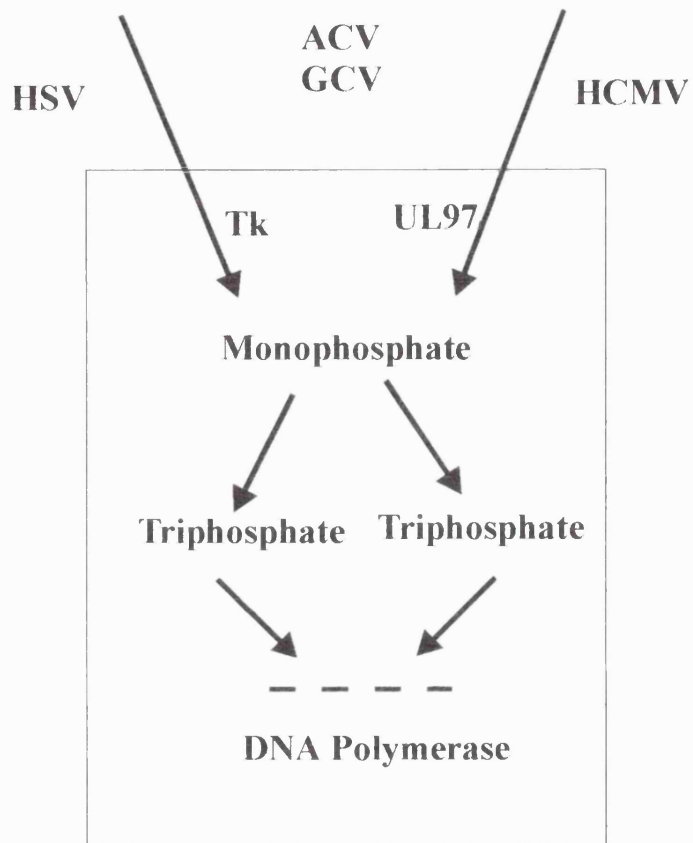


Figure 1.10 Mechanism of action of the acyclic deoxyguanosine analogues aciclovir and ganciclovir by herpes-induced phosphorylation.

1991, Winston *et al* , 1995). In bone marrow transplant recipients the effect of ganciclovir at reducing HCMV infection is more variable (Reed *et al* , 1990, Goodrich *et al* , 1993, Winston *et al* , 1993, Singhal *et al* , 1995).

Ganciclovir is mutagenic, carcinogenic and inhibits the growth of bone marrow progenitor cells as well as causing severe neutropenia. For this reason the administration of ganciclovir in severely immunocompromised individuals such as bone marrow transplant recipients must be carefully controlled. In addition to the adverse effects of ganciclovir, there is also the emergence of resistant strains, which appear primarily in patients with HIV. Ganciclovir resistance can occur by one of two mechanisms. The first and most common is due to mutations in the UL97 gene of HCMV where point mutations appear in many clinical isolates (Chou *et al* , 1995a, Chou *et al* , 1995b, Hanson *et al* , 1995). The second target for ganciclovir resistance is UL54, the polymerase gene of HCMV , which appear less frequently (Lurain *et al* , 1992, Tatarowicz *et al* 1992). Resistant strains have been isolated from transplant recipients with a much lower frequency, indeed, the prophylactic administration of ganciclovir in this population has not led to an increased incidence of ganciclovir resistance.

1.10.4 Foscarnet

Foscarnet a non-nucleoside analogue (known generically as trisodium phosphonoformate, with the product name Foscavir) is an effective anti-herpes virus agent that differs from aciclovir and ganciclovir as it is a pyrophosphate analogue (figure 1.9). The drug has broad spectrum antiviral activity against many of the herpes viruses including, HSV-1, HSV-2, VZV, EBV, HHV-6, HCMV and activity has also been reported against HIV and HBV. However, *in vitro* activity varies considerably due to the inefficiency of transport of this highly charged molecule across the cell membrane. The mechanism of action relies on non-competitive inhibition of the viral polymerase without the requirement of cellular enzymes for activation. In contrast, to the acyclic deoxyguanosine analogues, foscarnet does not incorporate into the growing DNA chain but blocks pyrophosphate binding on the viral DNA polymerase. Thus interfering directly with the pyrophosphate exchange from the dinucleotide triphosphates (dNTP's). The drug must be administered intravenously since oral bioavailability is poor at only 10-20% of the iv preparation. Following an iv dose of 60mg/kg, the peak plasma concentration of the drug is approximately 509 μ M (Wagstaff *et al* , 1994). The half-life is between 4.5-8 hours with the major route of elimination via the kidney. The main side effect is nephrotoxicity associated with raised creatinine levels in the urine which in extreme cases can lead to

acute renal failure.

Foscarnet is the treatment of choice for HCMV resistant to ganciclovir both in transplant recipients and in patients with HIV infection. Since the drug does not rely on activation by cellular enzymes, resistance only usually occurs in the viral DNA polymerase itself. Dual resistance to both foscarnet and ganciclovir occurs rarely, and *in vitro* experiments have shown that GCV resistant isolates are susceptible to foscarnet (Knox *et al* , 1991). As a first line treatment for HCMV retinitis in patients with HIV, ganciclovir remains the drug of choice, however, there is increasing administration of foscarnet as primary therapy for HCMV infection in the U.S.A (Studies of Ocular Complications of AIDS Research Group, 1992). Indeed, foscarnet has been successfully administered in conjunction with ganciclovir as alternating maintenance therapy in patients with AIDS (Peters *et al* , 1992, Dieterich *et al*, 1993, Jacobson *et al*, 1994). Clinical applications extend to the transplant population, with treatment for HCMV infections in patients undergoing bone marrow transplantation under investigation, which may be more favourable due to the reduced toxicity profile of foscarnet (Drobyski *et al* , 1991, Reusser *et al*, 1992, Bacigalupo *et al*, 1994).

1.10.5 Cidofovir

Another effective antiviral agent that has been approved for the treatment of HCMV retinitis in AIDS patients is cidofovir otherwise known as HPMPC with the chemical name { (S)-1-[3-hydroxy-2 (phosphonylmethoxy)-propyl]cytosine} the structure of which is shown in figure 1.9. The compound is an acyclic phosphonate nucleotide analogue which belongs to a family of phosphonylmethoxyalkyl derivatives of purines and pyrimidines. Cidofovir has activity against a wide range of herpes viruses including HSV-1, HSV-2, VZV and EBV (De Clercq *et al*, 1995). Indeed, HPMPC shows significant activity against clinical isolates of HSV and HCMV, with an IC_{50} value of between 0.06 - 0.08 μ g/ml for HCMV. Unlike ACV and GCV, cidofovir does not rely on activation by thymidine kinase or the homologous enzymatic activity of UL97. The compound is taken up by virus infected and non-infected cells and converted to its diphosphate derivative via phosphorylation which then acts as a competitive inhibitor for the natural substrate dCTP which in turn inhibits DNA polymerase (Ho *et al*, 1992). The selectivity of the cidofovir-diphosphate form of viral DNA polymerase is 25 - 50 fold greater than for the cellular DNA polymerase (Ho *et al*, 1992). The long lasting intracellular half-life (approximately 48 hours) allows infrequent dosing i.e once a week which distinguishes it from both ganciclovir and foscarnet which require administration several times daily to maintain an

antiviral effect.

Cidofovir is frequently used to treat HSV or HCMV in patients that have acquired resistance to either ganciclovir or foscarnet through the loss of thymidine kinase or UL97 functions. The drug can be administered either intravitreally for the treatment of established retinitis, or intravenously for systemic infection, as with other antivirals, the oral bioavailability is poor at only 5%. Treatment of HCMV retinitis in AIDS patients has been approved based on the studies by Lalezari *et al* (1997) and the SOCA ACTG trial (1997). The first of these studies showed a delay in retinitis progression after treatment with 5mg/kg once weekly for two weeks followed by maintenance therapy at once every other week. Intravitreal injections of 20 μ g at 5 to 6 week intervals for patients with HCMV retinitis may be an alternative to maintenance therapy (Kirsch *et al*, 1995a & 1995b). *In vitro* selected HPMPC resistant isolates show a single point mutation in the DNA polymerase gene, whereas isolates resistant to ganciclovir show mutations to both the DNA polymerase gene and the UL97 gene of HCMV. To date, however, there is no evidence of resistance due to treatment with HPMPC. At present, it is unclear whether HCMV resistance will develop following cidofovir therapy despite the potential to do so.

Aims of this Thesis

The objectives of the work carried out for this higher degree were:

1. To utilise qualitative and a fully quantitative competitive PCR assay for the detection of HCMV to monitor fluctuations in HCMV viral load from three immunocompromised patient groups namely; congenitally infected infants, renal transplant recipients and liver transplant recipients.
2. To analyse the use of these assays to provide information into the effect of antiviral therapy on HCMV viral load and to assess their ability to provide prognostic and pathogenic information in the immunocompromised host.
3. To correlate the longitudinal measurement of HCMV viral load with the clinical course of the patients.
4. To identify and quantify risk factors for HCMV disease in renal and liver transplant recipients.
5. To develop and optimise a fully quantitative reverse transcriptase PCR (QCRT-PCR) assay for the detection of HCMV gB mRNA.

Chapter 2.
Materials and Methods

2.1 Introduction

The following chapter describes methodologies used for the study of cytomegalovirus (HCMV) pathogenesis in the immunocompromised host. The patients analysed in this thesis include congenitally infected infants (chapter 3), renal (chapter 4) and liver transplant recipients (chapter 5). Longitudinal analysis was performed on clinical specimens from these patient groups using both qualitative and quantitative polymerase chain reaction (PCR) assays as described below. This technique was used to monitor fluctuations in viral load in response to antiviral therapy and to follow the clinical course of the patients. Also included are the methods used for the extraction of HCMV DNA from whole blood and the subsequent PCR amplification of HCMV DNA from blood and urine. Immunological techniques including the detection of HCMV antibodies in serum samples by immunofluorescence and methods for tissue culture are also described. In addition, cloning and DNA sequence techniques for the development of a quantitative competitive reverse transcriptase polymerase chain reaction (QC RT-PCR) are also detailed.

The development of the polymerase chain reaction (PCR) has permitted significant progression in molecular biology and diagnostics over the past decade. In the simplest form, PCR allows, by chemical means the increase of a specific nucleic acid relative to the others present in the sample of interest. The technique involves the use of two short sequences of complementary DNA or oligonucleotides that flank the gene or sequence of DNA of interest (also known as primer pairs). One primer is complementary to the 5' -3' end of the top strand of the DNA, the other being complementary to the 3' -5' end of the opposite bottom strand. The PCR reaction mixture including the primer pairs and sample containing the DNA is extended between the primers by repeated "cycles" of denaturation, primer annealing and finally enzymatic extension by *Taq* polymerase. This results in exponential accumulation of PCR products. *Taq* polymerase, unlike the first used Klenow fragment of *Escherichia coli* DNA polymerase I, is resistant to the higher temperatures required for the denaturation step. *Taq* polymerase, a heat dependent DNA polymerase that was first isolated from the bacterium *Thermus aquaticus*, has enhanced the versatility of PCR technology and limited the need for many manipulations and hence reduced contamination. This commercially prepared recombinant enzyme has the unique ability to extend the target DNA or cDNA from 5' -3' without 3' - 5' exonuclease activity.

Indeed, due to the high level of sensitivity, PCR is susceptible to the major problem of contamination which can only be eradicated by careful handling of each step in the

process and even by physical separation at each manipulation. For example, the initial step to prepare the reaction components is often prepared in a clean room devoid of any target DNA. The sample containing the target DNA is then added in a separate PCR set up area, and finally the reaction itself and resulting PCR products are processed and analysed in a separate post PCR area. All these precautions are necessary to prevent accumulation of contaminating DNA amplicons, which can lead to false positive results. The specificity of the reaction itself occurs as a result of several features. The first being the requirement for the annealing of two primers in the correct orientation within a span of less than approximately 10^4 nucleotides, except in the instance of "long PCR" where $>10^4$ nucleotides can be extended. The temperature at which the *Taq* itself is active i.e 72°C ensures only specific annealing of the chosen primer pairs. The use of an additional set of internal PCR primers in a subsequent amplification known as nested priming greatly reduces the probability of non-specific binding of primers and assures that only a single PCR product will arise.

In order to maximise the sensitivity and specificity of the PCR reaction it is necessary to optimise all the reaction parameters individually. Initially the primers chosen should contain a G-C content of between 40-60%, which should not contain any long stretches of a single base. The primer concentration itself is also critical i.e. if it is too high results in mispriming and generation of non-specific PCR products. Optimisation of the annealing temperature is especially important if total genomic DNA is used as the template, too low a temperature also results in non-specific priming. Primer specificity can be optimised further by titrating the concentration of dinucleotide triphosphates (dNTP's) and free magnesium ions in the reaction between a range of $20\mu\text{M}$ to $200\mu\text{M}$ and 0.5mM to 2.5mM respectively. The optimisation of the PCR reaction conditions for the qualitative and quantitative amplification of a 149 bp region of the glycoprotein B (gB) HCMV DNA used in this thesis are described in sections 2.2.2 and 2.4 respectively.

2.2 Methods

2.2.1 DNA Extraction from whole blood

DNA was extracted from whole blood samples using a commercially available ion-exchange chromatography QIAmp blood PCR kit supplied by Qiagen, UK. Each blood sample was processed in the same way, where a $200\mu\text{l}$ aliquot from a total of 10ml of whole blood was purified according to the manufacturers instructions. Briefly, $200\mu\text{l}$ of whole blood was treated with $200\mu\text{l}$ lysis buffer and $25\mu\text{l}$ of Qiagen proteinase

(19.3mg/ml) in a 1.5ml eppendorf tube. The mixture was vortexed and allowed to digest at 65°C for 30min prior to absorption onto the ion-exchange column in a 1.5ml eppendorf tube containing a silica matrix bed and centrifuged (Jouan MK14.11, Jouan, UK) at 6000g for 1min. The DNA that had absorbed to the matrix was then washed twice with 500µl Qiagen buffer AW containing guanidinium hydrochloride and the filtrate discarded. The DNA was finally eluted with 200µl sterile distilled water (SDW) pre-heated to 70°C followed by centrifugation at 6000g for 1min. If the sample was to be used directly in the PCR assay, it was heated to 90°C for 10min to inactivate the proteinase or alternatively was stored at -70°C until use. Samples of urine and CSF for the purpose of PCR were unprocessed. All clinical samples were tested qualitatively at neat and a 1:20 dilution.

2.2.2 Optimisation of Qualitative gB PCR

The PCR assay for the amplification of a 149bp fragment of HCMV gB had been previously described and fully optimised (Fox *et al*, 1992). However, due to a change in supplier of the PCR reagents from Bioline UK to Perkin Elmer PCR reagents manufactured by Roche Molecular Systems Inc (USA) this assay was re-optimised fully as described below under the same cycling conditions.

2.2.3 Primer optimisation of gB qualitative PCR

In order to determine the optimal conditions for the PCR reaction a primer titration was carried out using a fixed quantity of positive DNA template control of 10³ copies. The source of template was a plasmid clone of 149 bp of HCMV gB cloned into the pUC13 vector (Promega). A range of primer concentrations ranging from 50 - 500ng of each primer was amplified in GeneAmp 10X PCR buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM MgCl₂, 0.01%(w/v) gelatin), 1.25 units of Amplitaq Gold (5u/µl), 2mM MgCl₂ (25mM stock MgCl₂) with 200µM each dNTP (dATP, dGTP, dCTP, dTTP each at 6.25mM) in a total volume of 98µl made up with sterile distilled water (SDW). Negative water controls were included every fifth tube to assay for the presence of contaminating DNA. The PCR cycling conditions were the same for each reaction and cycling was performed on a Hybaid DNA thermal cycler as described below. The optimal primer concentration that gave the least background upon electrophoresis and visualisation of the agarose gel for the PCR reactions of the primer titration was found to be 100ng for each primer.

The sequence of the primers for the PCR assay to amplify a 149bp fragment of HCMV gB are given below:

5' GAG GAC AAC GAA ATC CTG TTG GGC A 3' (gB1)

5' GTC GAC GGT GGA GAT ACT GCT GAG G 3' (gB2)

The above sequences were derived from the laboratory strain AD169 corresponding to the nucleotides 81683 - 81707 (gB1) and 81559 - 81583 (gB2) (Chee *et al*, 1990). Each oligonucleotide primer was synthesised and HPLC purified commercially by Cruachem UK.

2.2.4 Magnesium titration

The PCR reaction was optimised further in GeneAMP 1XPCR buffer with varying MgCl₂ concentrations. The PCR reaction mix was made with the following concentrations of MgCl₂; 1, 1.5, 2, 2.5, 3, 3.5, and 4mM with 100ng each primer against 10³ copies of DNA template using the same cycling conditions as described in table 2.1. Negative and positive controls were also included to assay for false positives or system failure, where the positive control was 10³ copies of pUC13 containing the HCMV gB gene. The optimum MgCl₂ concentration which gave the least smearing without loss in sensitivity upon visualisation of the agarose gel was found to be 2mM.

2.3 Qualitative PCR amplification of gB HCMV DNA from clinical samples

This method was used for the amplification of a 149bp product from the gB gene of HCMV present in both unprocessed urine samples and DNA extracted from the whole blood of patients (Fox *et al*, 1992, Darlington *et al*, 1991, Kidd *et al*, 1993). Samples were stored in frozen aliquots at -70°C and thawed on ice prior to use in the PCR reaction. Both extracted DNA and urine (2µl) was added directly to 98µl of the PCR reaction mixture at both neat and a 1:20 dilution. The 1:20 dilution was used in an attempt to avoid the problem of PCR inhibition due to impurities in the clinical sample.

The PCR reaction was performed using a Hybaid thermal cycler in the optimised GeneAmp 1X PCR buffer consisting of 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl₂, 0.001% (w/v) gelatin, 200µM each dNTP, 1.25 units of Ampli Gold (5u/µl) and 100ng of each primer. Each PCR assay included a negative i.e water control every fifth tube and positive internal controls of 149bp fragment of gB cloned into plasmid pUC13 DNA at a concentration of 10³ and 10² copies. The resulting 149bp products were subsequently

analysed on 3% agarose gel containing ethidium bromide in 1X TBE buffer and visualised on a transilluminator with a PCR marker (50-1000bp, Promega).

Reagents for the PCR assay and analysis:

<i>dNTP</i> mixture:	6.25mM	dGTP (deoxyguanosine triphosphate)
(Promega)	6.25mM	dATP (deoxyadenosine triphosphate)
	6.25mM	dTTP (deoxythymidine triphosphate)
	6.25mM	dCTP (deoxycytosine triphosphate)

All of the reagents except for the addition of the target DNA were added in a PCR clean room with the use of aerosol resistant tips (Anachem UK) and with frequent changing of gloves to minimise the risk of contamination.

PCR reaction conditions for one tube:

10 μ l	10x GeneAmp 10X PCR buffer
8 μ l	MgCl ₂ (25mM)
3 μ l	dNTP mixture (200 μ M each)
1 μ l	gB1 (100ng)
1 μ l	gB2 (100ng)
0.25 μ l	AmpliTaq Gold(5u/ μ l)
74.75 μ l	SDW
(2 μ l	Target DNA sample)
98 μ l	Total.

The reaction mixture was aliquoted into a 0.5ml ependorf tube then overlaid with 100 μ l of molecular biology grade mineral oil (Sigma). For the analysis of multiple samples a master mix of reagents was prepared and 98 μ l aliquoted into each 0.5ml ependorf tube. The addition of the target clinical sample was carried out in a separate PCR set up area in a class II hood.

Table 2.1 PCR cycling conditions for qualitative HCMV gB PCR

Stage	Temperature (°C)	Duration	N°. Cycles
1.1	95	12 min	1
1.2	60	2 min	
1.3	72	2 min	
2.1	94	30 sec	35
2.2	60	30 sec	
2.3	72	30 sec	
3.1	94	30 sec	1
3.2	60	30 sec	
3.3	72	10 min	

2.3.1 Gel Preparation and electrophoresis

A 3% Agarose gel was prepared as follows for the analysis of the PCR products. 3g of agarose was weighed out into a Duran bottle and 100mls of 1X TBE buffer (see below) was added. The gel was melted in a 750W microwave oven on full power for approximately 3 minutes or until all of the agarose had melted. The gel was allowed to cool to approximately 50°C and 0.5µl of ethidium bromide (10mg/ml, Sigma) was added. The gel was poured into a gel frame with a 20 well comb, 15µl of each PCR product mixed with 5µl loading dye (30% glycerol 0.1% bromophenol blue in sterile distilled water) was added to each well together with a PCR marker (50-1000bp, Promega) to confirm the size of the amplicons. The gel was electrophoresed in 1X TBE at 150V for approximately 30 minutes, visualised on a transilluminator and photographed using Polaroid film.

10X TBE buffer:

432g	Tris base	(FW 121.1, Sigma)
220g	Boric acid	(FW 61.83, Sigma)
37.2g	EDTA	(FW 372.2, Sigma)

Made up to 4 litres with distilled de-ionised water.

The qualitative PCR assay was performed on samples at both a neat and 1:20 dilution as already mentioned. In the case of a positive at neat but not at 1:20 or vice versa, the PCR was repeated, if after this a band of 149bp was not seen the sample was classified as negative. Alternatively, if one of the dilutions gave a band of the correct size, this was scored as positive. Positive PCR results were re-analysed in a separate PCR reaction to confirm the initial result.

2.4 Quantitative-competitive gB PCR Assay

2.4.1 General considerations

The major use of this type of assay to date is to monitor viral replication. Quantitative PCR technology is a powerful tool providing information into the pathogenesis of viruses by determining the viral load at a particular time point, or more informatively, by performing longitudinal analysis. Methods for quantitation are based on the comparison of two amplified signals one of which is of known quantity. The main objectives are to develop a fully quantitative assay over a broad dynamic range to allow quantitation of low

and high copy numbers of DNA. The two sequences for comparison in the quantitative assay i.e the internal control and target sequences, should be as closely related to each other as possible. In addition the control sequence should be added to each PCR reaction to rule out the possibility of tube to tube variation.

Several strategies for differentiation between the control and unknown target sequence have been employed including size differentiation by insertion or deletion of the internal control DNA, amplification with different primers of endogenous sequences that are functionally related, or the introduction of a unique restriction site into the control sequence itself. The latter of these strategies presents the best scenario since the difference between the target and the internal control sequence is limited to a few bases, and can be achieved by simple mutagenesis. The co-amplification of an internal control sequence with a unique restriction site together with the target DNA is the method of choice for this thesis. In order to determine the limits of a quantitative assay, the dynamic range must first be determined by production of a calibration curve, whereby the control and the target sequences are compared. Consideration of dynamic range is based on the fact that the PCR product itself accumulates exponentially up to the plateau phase of the reaction.

The quantitative assay used in this thesis is based on the co-amplification of a known copy number of internal control with the target DNA present in the clinical sample which has been described previously in detail (Fox *et al*, 1992). The internal control harbours a unique restriction site for the enzyme *Hpa*1 by mutagenesis of the wild type sequence from the original GG to TT within the gB gene of HCMV. This mutant sequence was then sub-cloned into a pUC13 vector (Pharmacia) and purified as a source of internal control DNA. The primers for the PCR are identical to those used for the qualitative assay described above with the exception that gB2 is 5' OH labelled with [γ -³²P]ATP to allow detection following the PCR reaction. Quantitation is achieved following digestion of the PCR product with *Hpa*1 which cleaves only the internal control sequence into 77 and 72bp fragments leaving the target DNA in the sample of interest undigested. The digestion products are separated by polyacrylamide gel electrophoresis (PAGE) and subjected to autoradiography and scanning densitometry. Since a known quantity of internal control is included in each PCR reaction, a direct comparison of the intensity of the target DNA with the cleaved internal control allows the copy number determination of unknown target to be calculated. To ensure accuracy in this calculation, three tubes containing different known quantities of control sequence but the same amount of the

unknown target present in the clinical sample are assayed in parallel.

2.4.2 Phosphorylation of primer gB2

Initially to allow detection of the PCR products, one of the primers was 5' OH end labelled with [γ - 32 P]ATP using T4 polynucleotide kinase (PNK). This enzyme catalyses the end labelling of 5' termini of DNA or RNA by transferring the γ -phosphate of ATP to the 5' OH group of the primer. The gB2 primer was 5' phosphorylated by adding the reaction components in the order stated below at 37°C for 30min, the reaction was stopped by placing it at -70°C until use.

<u>Labelling Reaction</u>		<u>Forward Buffer</u>	
2 μ l	gB2 (250ng/ μ l)	350mM	Tris-HCL
20 μ l	5X Forward Buffer (Gibco BRL)	50 mM	MgCl ₂
4 μ l	PNK(10u/ μ l) (Gibco BRL)	500mM	KCL
10 μ l	[γ - 32 P]ATP(100 μ Ci) (Amersham)	5mM	2-mercaptoethanol
66 μ l	SDW		
100 μ l	Total		

Once the primer had been labelled, the PCR reaction and cycling conditions were performed as for the qualitative PCR in section 2.3 with the exception that 1 μ l (5ng/ μ l) of labelled gB2 primer was added. The target DNA in this case consisted of known copy numbers of both the internal control and wild type sequence, both sequences were subsequently co-amplified in the same tube. Each tube contained the same copy number of the internal control, with the following range for the wild type sequence; 10⁵, 10⁴, 10³, 10² and 10 copies.

Quantitative PCR reaction mixture for one tube:

10 μ l	10X GeneAmp 10X PCR buffer
8 μ l	MgCL ₂ (25mM)
3 μ l	dNTP mixture (200 μ M each) (Promega)
1 μ l	gB1 (100ng)
1 μ l	gB2 (100ng)
1 μ l	gB2* ([γ - 32 P]ATP 5ng/ μ l)
0.25 μ l	AmpliTaq Gold (5u/ μ l) (Amplitaq gold)
71.75 μ l	SDW
(2 μ l	Internal control DNA sample)
(2 μ l	Wild type control DNA)
100 μ l	Total

The reaction mixture was overlaid with 100 μ l of molecular biology grade mineral oil (Sigma).

Note: As with the qualitative PCR, the internal and wild type sequences were added under the oil in a separate PCR set up area as were the negative water controls added every fifth tube to assess for contamination problems.

2.4.3 12% PAGE electrophoresis of PCR products

After cycling, the PCR products were electrophoresed on a 3% agarose gel as described above for the qualitative PCR in section 2.3.1. Following confirmation that the reaction had worked, 10 μ l of each of the products were digested with 1 μ l of *Hpa*1 (5units/ μ l) in 2 μ l 10X buffer (200mM Tris-HCl pH 7.4, 50mM MgCl₂, 500mM KCl) made up to 20 μ l with 7 μ l SDW at 37^oC for 2 hours. After digestion the products were mixed with 5 μ l loading dye and separated on a 12% PAGE gel (6ml 40% *bis*-polyacrylamide, 2ml 10X TBE, 140 μ l 10% w/v ammonium persulphate (APS), 14 μ l N,N,N',N' tetramethylethylenediamine (TEMED)) run in 1XTBE buffer at 45mA for approximately 1.5 hours, or until the dye had run off the bottom of the gel. The gels were stained in 50ml 1XTBE containing 5 μ l (10mg/ml) ethidium bromide solution for approximately 5 minutes or until the DNA could be visualised using a transilluminator and subsequently photographed. The wet gel was covered with cling film, placed in an X-ray film cassette and exposed to autoradiographic film (Hyperfilm-MP, Amersham UK). The film was exposed for anything from 2-24 hours depending upon the activity date of the radioactive label. Scanning densitometry was performed on the autoradiographs with a Shimadzu CS 9001 PC dual wave scanning densitometer and the copy number from each sample calculated.

2.5 Tissue Culture

2.5.1 Tissue culture of laboratory strain AD169

The laboratory strain of HCMV, AD169 was cultured in human embryo lung cells (HEL) according to standard procedures. Initially HEL cells were grown to 80-90% confluency in 30ml growth media (Gibco MEM media supplemented with 10% foetal bovine serum (FBS) and penicillin /streptomycin (Pen/Strep) 100u and 10000u respectively) in a T175 tissue culture flask. The media was removed from the cells, the cell sheet washed once with 10ml maintenance media (Gibco MEM media supplemented with 5% FBS and Pen/Strep). The maintenance media was removed and 5ml of stock AD169 at an moi of 0.01 was inoculated onto the cell sheet and allowed to adhere to the cells for one hour

at 37°C. The viral inoculum was removed, the cell sheet was washed once in maintenance media and replaced with 10ml fresh maintenance media and the cells were incubated at 37°C. When cytopathic effect (cpe) became visible the supernatant containing the virus was removed. The cells were centrifuged at 1000g to remove the cell debris and the supernatant was frozen at -70°C, another 10ml maintenance media was added to the cells and the cells were incubated at 37°C for a further 24 hours. Collection of the viral stock supernatant from the cells described above was continued until there were no more adherent cells visible, the titre of these stocks was then determined by plaque assay.

2.5.2 Plaque Assay

Initially, 48 well plates were seeded with 1×10^5 HEL cells in 1ml growth media per well and grown to 90% confluency at 37°C in a 5% CO₂ incubator overnight. Dilutions of the supernatants containing virus were made in half log₁₀ steps ranging from 10⁻¹ to 10⁻⁷ in maintenance media. Media was removed from the cells and the cell sheet washed once with maintenance media. Triplicate wells were inoculated with 100µl of each virus dilution and incubated at 37°C for one hour. The viral inoculum was removed and each well was washed with 1ml of maintenance media. The cells were overlaid with 2ml methyl cellulose overlay (MCO) per well, which was prepared by adding 50ml of media (10ml 10 X MEM Gibco, 5ml FBS, 3ml 7.5%NaHCO₃ Gibco, 0.5ml L-glutamine Gibco, 1ml Pen/Strep, 30ml L-15 media Gibco) to 50ml 2% methyl cellulose (w/v, Sigma) made up in SDW. Plates were incubated at 37°C in 5%CO₂ for between 10-14 days or until the appearance of cpe. The wells were then fixed with 1ml formalin/saline solution (4g NaCl FW 58.44 Sigma, 50ml formalin FW 30.03 Sigma, 450ml SDW) for 30 min. After fixing, the wells were washed thoroughly with distilled water until the MCO was completely removed. The plaques were stained with 0.5 ml methylene blue stain per well for 30 min (0.15g methylene blue in 500ml SDW), the stain removed and the plates washed in distilled water to remove excess stain and allowed to dry. Plaques were counted in triplicate wells for each virus dilution and the titre for each virus stock calculated

2.5.3 TCID₅₀ assay

The quantity of virus in a sample is often directly related to the effect that virus may have on the patient as is the case for HCMV in congenitally infected infants where the more virus excreted in the urine, the worse the prognosis for the child. This method allows the quantity of virus present in urine to be determined by tissue culture, where the infectious

dose to give 50% (TCID₅₀) infection in human embryo lung (HEL) cells is calculated.

2.5.4 Preparation of tubes for TCID₅₀ assay

Initially, 3 tubes of HEL cells for each urine sample dilution were prepared and grown to confluency in growth media, where each tube contained approximately 1X10⁶ cells. For each sample a dilution series, (ranging from 10⁻¹ to 10⁻⁸ in half log₁₀ steps) of urine was made in maintenance media. The growth medium was removed from the cells and 200µl of each sample dilution was inoculated in triplicate into each tube. Negative and positive controls were also included for each sample inoculated with either 200µl of maintenance media or 200µl of neat urine sample respectively. After 1 hour incubation at 37°C the viral inoculum was removed and the tubes were re-fed with 1.5 ml maintenance media. Tubes were incubated at 37°C and re-fed each day with 1.5ml fresh maintenance media until cpe was observed at the lowest dilution (approximately 21 days for HCMV). The TCID₅₀ value was calculated for each urine sample under test, where any tube showing cpe was scored as positive.

2.5.5 Calculation of 50% end-point for virus titration

The accumulated values for infected tubes were calculated by adding the total number of infected tubes from the highest dilution that showed cpe to the lowest. Accumulated values for tubes without cpe i.e healthy tubes, were calculated by adding the total number of healthy tubes from the lowest dilution to the highest. Initially the proportionate distance (PD) of the 50% end point is calculated.

$$PD = \frac{(\% \text{ mortality at dilution next above } 50\%) - 50\%}{(\% \text{ mortality at dilution next above } 50\%) - (\% \text{ mortality at dilution next above } 50\%)}$$

next multiply by the negative log of the dilution factor i.e

$$a \times (-1.0) = -a$$

Then the corrected PD is added to the negative log of the dilution next above 50% mortality to give the 50% end point i.e (-a) + (next dilution factor at which 50% mortality occurs e.g 10⁻⁵) = -5.a

Therefore, the 50% end point is 10^{5.a}. In other words the urine sample must be diluted 1 in 10^{5.a} to achieve 50% infection in the tubes.

2.6 Insect cell culture

2.6.1 General considerations

This eukaryotic expression system for expressing foreign genes was chosen to express gB for the purpose of detection of antibodies in human sera by immunofluorescence. Baculovirus vectors are commonly used as expression systems due to their ability to produce large quantities of protein with limited post-translational modification. These large double stranded DNA viruses are named according to the type of host that they infect and the type of occlusion structures that are made upon infection. The baculovirus exploited for the expression of the genes in this section normally infect the alfalfa looper moth, *Autographa californica* (AcNPV). Upon infection intracellular inclusions are formed in excess quantities that consist of virions encased in crystals composed of a single protein (polyhedrin, M_r 29,000). The production of this protein is driven by an extremely active promoter, which can in turn be used to drive an inserted gene of interest by removal of the non-essential polyhedrin gene. The recombinant baculovirus expressing gB of HCMV were inserted into a baculovirus transfer vector and then co-transfected with full length baculovirus DNA into cultured insect cells, upon recombination of the genes at a specific locus, the recombinant viruses were selected, purified and amplified to produce a pure culture of recombinant virus. The recombinant viruses expressing gB and wild type virus were all a kind gift from Professor W.B Britt (Alabama, USA).

2.6.2 Growth of Baculoviruses in culture

All baculovirus strains including recombinant and wild type viruses were maintained in SF21 (Invitrogen) insect cells according to standard protocols. Initially the SF21 cells were grown to 50% confluency in a T75 tissue culture flask in 10ml of complete TC-100 media (Gibco BRL, supplemented with 10% FBS and Pen/Strep 100u and 1000u respectively) at 28^oC. The media was removed from the cells and the appropriate virus inoculum added at a multiplicity of infection (moi) of 10 in 2ml of complete media TC-100. The virus inoculum was allowed to adhere to the cells for one hour at room temperature and was then replaced with 10ml fresh complete media. For the preparation of high titre stocks, the virus supernatant was collected by removing the cells from the flask and centrifuging the cell suspension at 1000g. The supernatant was passaged through SF21 cells at least five times, and the supernatants from each passage were stored at 4^oC, the titre of each stock was determined by plaque assay.

2.6.3 Baculovirus plaque assay

Initially 2ml tissue culture dishes were seeded with 5×10^5 SF21 cells in 2ml complete TC100 media and allowed to grow to 50% confluency i.e after approximately 24 hours at 28°C. Media was removed from the cells, and each dish inoculated in triplicate with 100 μ l of the virus stock diluted in log₁₀ steps in complete TC-100 media i.e 10^{-1} , 10^{-2} and so on to 10^{-8} . A negative control inoculated with 100 μ l complete TC-100 media was also included. The virus inoculum was removed after incubation at room temperature for one hour. 3% w/v agarose in SDW (molecular grade type VII low melting temperature, Sigma) was melted, cooled to 45°C and diluted to a final concentration of 1.5% with complete TC-100 media. 1ml of the agarose overlay was added to the cells and allowed to set for approximately 30 minutes at room temperature then 1ml of complete media was added to each dish. The dishes were incubated in a humidified chamber at 28°C for 5-6 days or until the negative uninfected control cells became confluent. Plaques were stained with 2ml neutral red per well (0.025% w/v in sterile PBS, Sigma) for two and a half hours at room temperature, the stain removed and plates placed in the dark for 24 hours at room temperature. The plaques were counted and the viral titre of each virus stock calculated.

2.6.4 Indirect Immunofluorescence (IFA) for gB and gH in sequential serum samples

This technique was used to detect antibodies to the glycoprotein B (gB) of HCMV in sequential serum samples from liver transplant recipients. Slides were prepared from recombinant baculovirus vectors expressing gB as described below. Sera was tested and the final end point dilution of sera to give positive immunofluorescence determined using a Zeiss immunofluorescence microscope.

2.6.5 Slide preparation

Sf21 cells were grown by standard protocols to 50% confluency and infected with either recombinant baculovirus expressing gB or non-expressing wild type baculovirus at an moi of 10. Each flask contained approximately 5×10^6 cells, the negative control flask consisted of uninfected SF21 cells. The viral inoculum was left to adhere to the cells at room temperature for one hour and then replaced with 10ml fresh complete TC-100 media. The viral cultures and negative control uninfected cells were incubated at 28°C for 48 hours to allow for expression of the proteins. The cell monolayers were gently removed, cells from the infected and non-infected cultures were harvested by centrifuging at 1000g for 5 min. Cells were washed with 10 ml PBS three times to remove any serum proteins and resuspended in 10 ml fresh sterile PBS. The cell count from

each culture resuspended in 1ml PBS was determined using a haemocytometer. The cell concentration was adjusted to 2×10^6 cells /ml. 12 well PTFE coated slides (poly-tetra-fluoro-ethylene, Hendley Essex U.K) were divided into two sections consisting of the top row of 6 wells for the recombinant virus and the bottom row of 5 wells for the uninfected negative control wells and one well for the wild type virus was also included. $15 \mu\text{l}$ of the cell suspensions were spotted onto each well and allowed to air dry for 1 hour at room temperature. The slides were fixed in ice-cold 100% acetone at -20°C for ten minutes and stored at -20°C until use.

2.6.6 IFA assay for anti-gB antibodies

The IFA assay was performed on sera from patients as follows to determine the presence or absence of antibodies to gB. The sera were diluted in doubling dilutions in sterile PBS initially at a 1:30 dilution. $15 \mu\text{l}$ of each dilution was spotted onto each well coated with the appropriate recombinant antigen (i.e either gB or wild type) as well as the corresponding uninfected well. A positive control well for each slide was also included and consisted of $15 \mu\text{l}$ of a mouse monoclonal antibody (7-17) against gB (dilution factor 1:40). Slides were incubated at 37°C for 40 minutes in a humidified chamber and washed once with 1% bovine serum albumin (w/v, Sigma) in PBS, followed by two washes in PBS alone. The secondary antibody fluorescein isothiocyanate-conjugated (FITC) rabbit anti human IgG F(ab')₂ conjugate was diluted 1:40 in PBS and $10 \mu\text{l}$ was spotted onto all wells except for the positive controls where the secondary antibody was goat anti-mouse IgG F(ab')₂ FITC conjugate which was also diluted 1:40 in PBS. Slides were incubated for 40 minutes at 37°C in a humidified chamber, washed as described above and allowed to dry at room temperature in the dark for approximately 30 minutes. Once dry, the slides were mounted with "Citifluor" (UKC, Canterbury) mounting fluid and sealed with a coverslip to preserve the fluorescence. Slides were visualised with a Zeiss immunofluorescence microscope, the end-point was scored as the final dilution at which positive fluorescence could be seen.

2.7 Development of a quantitative competitive reverse transcriptase PCR (QCRT-PCR)

The following section describes the methodologies used for the development of a quantitative competitive reverse transcriptase PCR assay (QC RT-PCR) for the detection of HCMV in blood and tissue samples.

2.7.1 Preparation of the gB 149bp insert for cloning into pT3/T718U vector

The original pUC13 plasmids containing the 149bp inserts of either wild type gB or control gB with the mutated *Hpa1* restriction site were digested with EcoR1 and Hind III in a double digest to remove the insert from the multiple cloning site. 10 μ l of pUC13 plasmid DNA (500ng/ μ l) with either sequence of the gB insert was digested with 1.0 μ l EcoRI (10 units/ μ l) and 1.0 μ l HindIII (10 units/ μ l), 3 μ l 10X buffer B (10mM Tris-HCl, 100mM NaCl, 5mM MgCl₂, 1mM β -mercaptoethanol) made up to a final volume of 30 μ l with 16 μ l SDW. The reaction mixture was digested at 37^oC for 2 hours. 2 μ l of each digestion was removed, 8 μ l of SDW and 5 μ l of loading dye was added, the products were then analysed on a 1% agarose gel in 1XTBE buffer together with 1 μ g λ HindIII/EcoRI marker and 1 μ g PCR marker (Promega 100-1000bp).

2.7.2 Preparation of pT3/T718U vector

The vector pT3/T718U (Pharmacia) was digested with EcoR1 and Hind III for 2 hours at 37^oC to open the multiple cloning site to allow unidirectional insertion of the gB DNA fragments by cohesive end ligation. 10 μ l of the pT3/T7 18U (100ng/ μ l) vector was digested with 1.0 μ l EcoRI (10 units/ μ l) and 1.0 μ l HindIII (10 units/ μ l), 3 μ l 10X buffer B (10mM Tris-HCl, 100mM NaCl, 5mM MgCl₂, 1mM β -mercaptoethanol) made up to a final volume of 30 μ l with 16 μ l SDW. The reaction mixture was digested at 37^oC for 2 hours. 2 μ l of the digestion reaction was removed together with 2 μ l of the uncut vector (diluted 1:10), 8 μ l of SDW and 5 μ l of loading dye was added and the DNA analysed on a 1% agarose gel in 1XTBE buffer together with 1 μ g λ HindIII/EcoRI marker and 1 μ g PCR marker (Promega 100-1000bp) to verify complete digestion of the vector.

2.7.3 Purification of pT3/T718U and the gB wild type and control inserts

Once the sizes of the inserts and the vector had been confirmed by gel electrophoresis, the remainder of the digests from section 2.7.1 and section 2.7.2 were purified from a 1% low melting point (1% w/v, Sigma) molecular biology grade low melting point agarose in 1XTBE, and used for ligation. The pT3/T718U vector DNA and inserts were analysed on a 1% low melting point gel at 75 volts in 1XTBE buffer for approximately 45min, or until when visualised the insert fragment had separated sufficiently from the pUC13 vector. The gel fragments of interest were excised from the gel using a scalpel under short wave length U.V to minimise DNA mutagenesis. DNA was purified from the gel using the Gene Clean II kit (Bio, 101, USA) according to the manufacturer's instructions. Briefly the gel fragment was weighed, and 1/2 volume of TBE modifier buffer added followed by 4.5 volumes of sodium iodide (NaI) stock solution. The fragments were melted at 55^oC for 5

minutes, 10 μ l of GLASSMILK suspension was added mixed and incubated on ice for 5 minutes to allow the DNA to bind the silica matrix. The GLASSMILK was pelleted at 13,000g for one minute and the supernatant removed. The pellet was washed three times with 700 μ l NEW WASH and the DNA eluted from the matrix by incubation at 55 $^{\circ}$ C for 3 minutes in 25 μ l T.E (pH 8.0). A 2 μ l aliquot of each of the purified 149bp gB inserts and the vector DNA was analysed on a 1% agarose with 1 μ g λ HindIII/EcoRI marker and 1 μ g PCR marker (Promega 100-1000bp) to confirm the purity and size of the DNA.

2.7.4 Cohesive end ligation of the 149bp gB inserts into pT3/T718U vector

100ng of the 149bp gB inserts were ligated into 10ng of the vector (as a 1:1 ratio) in the presence of 1.25 units of T4 DNA ligase (Northumbria), 1mM ATP (Amersham) in 1 μ l 10X ligation buffer (0.5M Tris-HCl pH 7.5, 100mM MgCl₂, 100mM DDT, 500 μ g/ml bovine serum albumin) made up to a total volume of 10 μ l with SDW. Control ligations of the linearised vector alone and the insert alone with all ligation reaction components were also prepared. Ligation reactions were incubated at 16 $^{\circ}$ C overnight. 10 μ l of each ligation reaction was then used to transform 100 μ l of competent *Escherichia coli* (*E. coli*) JM109 cells as described.

2.7.5 Preparation of transformation competent *E. coli* JM109 cells

JM109 cells (Pharmacia) carrying the F' episome with the following genotype were used for all transformation experiments:

e14⁻(MrcA⁻) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_k m_k⁺) *supE44 relA1*Δ(*lac-proAB*) [F'
*traD36 proAB lac^FZ*ΔM15]

A single colony from a Y_a agar plate (0.5% (w/v) yeast extract (Sigma), 2% (w/v) tryptone (Oxoid), 20mM MgSO₄, 1.4% agar (Sigma) the pH of the agar was adjusted to 7.6 with KOH prior to autoclaving) streaked with a stock culture of *E. coli* JM109 cells was picked aseptically and inoculated into 7ml YB media (0.5% (w/v) yeast extract (Sigma), 2% (w/v) tryptone (Oxoid), 20mM MgSO₄, and the pH of the media adjusted to 7.6 with KOH prior to autoclaving), and incubated for 2-3 hours at 37 $^{\circ}$ C in a shaking incubator (250 rpm) until the OD₅₅₀ reached 0.3. 5ml of the culture was used to inoculate 100ml sterile YB media (pre-warmed to 37 $^{\circ}$ C) in a conical flask, the culture was shaken (250 rpm) at 37 $^{\circ}$ C for approximately 2 hours or until the OD₅₅₀ reached 0.48. The cells were chilled on ice for 20 minutes and centrifuged in 30ml Falcon tubes at 6000g for 5min at 4 $^{\circ}$ C. JM109

cells were resuspended in 2/5 volume i.e 40ml of ice cold Tfb1 buffer (30mM potassium acetate, 100mM RbCl₂, 10mM CaCl₂, 50mM MnCl₂, 15% (v/v) glycerol, and the pH adjusted to 5.8 with 0.2M acetic acid prior to sterilisation by filtration) and left on ice for 20 minutes. Cells were centrifuged again at 5000g for 5 minutes at 4^oC and resuspended in 4ml ice cold Tfbll buffer and left on ice for a further 20 minutes (10mM Mops, 10mM RbCl₂, 75mM CaCl₂, 15% (v/v) glycerol, the pH adjusted to 5.8 with KOH prior to sterilisation by filtration). The competent JM109 cells were aliquoted into pre-cooled ependorf tubes in 200 μ l aliquots snap frozen in a methanol/dry ice bath and stored at -70^oC until use.

2.7.6 Transformation of competent *E.coli* JM109 cells with recombinant pT3/T718U vectors

Plasmid recombinant clones were prepared by transforming *E.coli* JM109 cells followed by mini-scale preparation of plasmid DNA described in section 2.7.7. 600 μ l of competent JM109 cells were thawed on ice, once the cells had just thawed 100 μ l of cells were aliquoted into a 1.5ml tube i.e one tube per ligation reaction . 10 μ l of each ligation reaction from section 2.7.4 was added to the cells and incubated on ice for 30 minutes followed by a heat shock at 42^oC for 90 seconds. The tubes were placed on ice for 1 minute to cool, 400 μ l of γ B (pre-warmed to 37^oC) was added to each tube and incubated at 37^oC for 1 hour. 100 μ l aliquots of the transformed cells were plated out onto luria broth (LB) agar plates (0.5% (w/v) NaCl (Sigma), 0.5% (w/v) yeast extract (Sigma), 1.0% (w/v) tryptone (Oxoid) 1.5% (w/v) agar (Oxoid), and the pH adjusted to 7.5 with NaOH prior to sterilisation by autoclaving the agar was left to cool) supplemented with 50 μ g/ml ampicillin, 40 μ g/ml 5-bromo 4-chloro 3- indolyl- β -D-galactoside (X-Gal) and 46 μ g/ml isopropyl- β -D-thiogalactopyranoside (IPTG) to allow selection of the recombinant clones. Colonies with the inserted DNA i.e white colonies were selected against the non-recombinants (blue colonies).

2.7.7 Mini-preparation of plasmid DNA from overnight bacterial cultures

The white putative recombinant colonies were picked and inoculated into 5ml LB broth containing 50 μ g/ml ampicillin and incubated in a shaking incubator at 37^oC overnight. The selected colonies were also streaked onto LB agar plates supplemented with 50 μ g/ml ampicillin and stored at 4^oC as a stock. The bacterial suspension was used to make glycerol stocks, 200 μ l (20%) of glycerol (Sigma) was added to 800 μ l of cells which was vortexed and snap frozen in a methanol/dry ice bath and stored at -70^oC. The remainder of the overnight bacterial cultures were used for DNA purification using the Wizard Plus

Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Briefly 1.5 ml of each overnight culture was centrifuged at 10,000g in a bench top microcentrifuge for 5 minutes and the supernatant discarded. 200 μ l of cell resuspension solution (50mM Tris-HCl pH 7.5, 10mM EDTA, 100 μ g/ml Rnase A) was added and the tubes vortexed vigorously to ensure complete resuspension of the cells, 200 μ l of cell lysis solution was then added and the tubes inverted several times to ensure complete lysis, 200 μ l of neutralisation solution (1.32M potassium acetate) was added and the cell lysate centrifuged at 10,000g for 5 minutes in a microcentrifuge. The cleared lysate was carefully removed and added to 1ml Wizard Minipreps DNA Purification resin, the tube was inverted several times to ensure binding of the DNA and pipetted into a 2ml syringe barrel (Sterilin) devoid of the plunger, attached to a Wizard Minicolumn. The syringe plunger was carefully inserted into the barrel and used to push the slurry into the column the flow through was discarded, 2 ml column wash (80mM potassium acetate, 8.3M Tris-HCl, pH 7.5, 40 μ M EDTA, 55% (v/v) ethanol) was pipetted into the barrel and pushed through. The minicolumn was placed into a 1.5ml ependorf tube and centrifuged at 10,000g for 3 minutes to dry the resin. The DNA was eluted from the column by the addition of 50 μ l T.E (pH8.0), and centrifugation at 10,000g into a clean 1.5 ml ependorf tube.

2.7.8 Screening of plasmid clones by restriction enzyme digestion

The isolated DNA from the plasmid clones was analysed by restriction enzyme digestion to check for the presence of the inserted gB 149bp fragment. 10 μ l of the plasmid DNA isolated from section 2.7.7 was digested with 0.5 μ l *EcoR1* (5u/ μ l) and 0.5 μ l *Hind III* (5u/ μ l) in 2 μ l 10X buffer B (10mM Tris-HCl, 100mM NaCl, 5mM MgCl₂, 1mM β -mercaptoethanol) made up to a final volume of 20 μ l with 7 μ l of SDW at 37^oC for 2 hours. 10 μ l DNA from each clone was also digested with 0.5 μ l *Hpa1* (5u/ μ l) in 2 μ l 10X buffer (200mM Tris-HCl pH 7.4, 50mM MgCl₂, 500mM KCl) made up to 20 μ l with 6.5 μ l SDW and digested at 37^oC for 1 hour to differentiate between wild type and mutated gB. The digestion products were analysed on a 1% agarose gel in 1XTBE buffer as described in section 2.3.1.

2.7.9 Plasmid sequencing of recombinant pT3/T718U with gB 149bp inserts

Plasmid DNA containing the 149 bp gB inserts from section 2.7.7 was sequenced using Sequinase Version 2.0 T7 DNA polymerase (United States Biochemical,USB) with universal forward and reverse primers. The sequencing reaction was performed on five clones of wild type and control gB insert as determined by the restriction digests in

section 2.7.8 using the protocol as outlined by USB. 5 μ g of DNA (up to 20 μ l) was denatured with 5 μ l 1M NaOH, 1mM EDTA (pH 8.0) in a total volume of 25 μ l made up with SDW at room temperature for 10 minutes. A CL-6B sepharose (Sigma) column was prepared and equilibrated with an equal volume of T.E (pH 8.0). A small quantity of glass beads (425–600 μ m diameter, Sigma) was placed into a 0.5 ml ependorf tube with a hole pierced in the bottom, and overlaid with 700 μ l of the CL-6B sepharose. This was placed inside a 1.5ml ependorf tube with a hole pierced in the bottom and centrifuged at 1400g for 5 minutes in a Beckman TJ-6 bench top centrifuge. The denatured DNA was placed onto the column and the 1.5ml ependorf replaced with an intact tube and centrifuged at 1400g for 5 minutes. The collected denatured DNA was divided into two 7 μ l aliquots, one aliquot was annealed to the sense (+, forward) primer, the other to the antisense (-, reverse) primer. 1 μ l (50ng/ μ l) of either primer was added to 7 μ l DNA in 2 μ l 5X sequenase buffer (200mM Tris-HCl pH 7.5, 100mM MgCl₂, 250mM NaCl, USB) incubated at 37^oC for 15 minutes allowed to cool to room temperature and chilled on ice. 2 μ l nucleotide labelling mix (1.5 μ M each dCTP, dGTP dTTP), diluted 1:5 in SDW, 1 μ l DDT (0.1M solution), 0.5 μ l [α -³⁵S]d ATP (5 μ Ci, Amersham UK), 2 μ l Sequenase enzyme (3 units) diluted 1:8 with enzyme dilution buffer (10mM Tris-HCl pH7.5, 5mM DTT, 0.5mg/ml BSA) was added to the annealed DNA and incubated at room temperature for 5 minutes. 2.5 μ l of each ddNTP, i.e ddATP, ddGTP, ddCTP, ddTTP was added to four ependorf tubes with a set of four ddNTP's for each forward and reverse reaction per clone sequenced. 3.5 μ l of the annealed reaction mixture was added to each of the four ependorf tubes containing the ddNTP's prewarmed to 37^oC and incubated at 37^oC for 5 minutes before the addition of 4 μ l stop solution (95% (w/v) formamide, 20mM EDTA pH8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF). The samples were heated to 90^oC for 5 minutes and stored at -20^oC until analysis by polyacrylamide denaturing urea gel electrophoresis.

2.7.10 Analysis of DNA sequencing reactions by Polyacrylamide Urea Gel Electrophoresis

The complete sequencing reactions from section 2.7.9 were analysed on a 6% urea polyacrylamide wedged sequencing gel. The gel was prepared by dissolving 75g urea (molecular biology grade, Sigma) in 22.5 ml 40% polyacrylamide solution (38% acrylamide, 2% bisacrylamide, BDH), 15 ml filtered 10X TBE and 52.5 ml SDW on a heated stirrer at 50^oC for 1 hour or until the urea had completely dissolved. The mixture was then chilled on ice for approximately 20 minutes, 900 μ l of 10% (w/v) APS (Sigma), 80 μ l TEMED (Sigma) was added and mixed thoroughly. The gel mix was poured between

two 33 x 43 cm (5mm thick) glass plates, (Gibco) which had been thoroughly cleaned with 70% ethanol and the shorter plate had been siliconised with dichlorodimethylsilane (Sigma). The gel plates were wedged at the bottom with two spacers (20 x 0.4mm), two 24 well shark toothed combs (Sigma) were placed in the top of the gel before it had set and 1XTBE was used to remove any unpolymerised acrylamide or any residual urea prior to loading. The gel was allowed to set at room temperature for 30 minutes and pre-warmed by running in 1XTBE buffer at 40 volts for 30 minutes .

5 μ l of the sequencing reactions labelled "G", "A", "T", "C" for the forward and reverse reaction from section 2.7.9 were loaded in the order stated into adjacent wells of the gel for each clone and subjected to electrophoresis at 75W until the bromophenol dye was approximately 3cm from the bottom of the gel ("long run"). The remaining 5 μ l of each sequencing reaction was loaded into a different set of 4 adjacent wells as a second loading and run until the bromophenol dye was approximately 3cm from the bottom of the gel ("short run"). The gel plates were separated leaving the gel attached to the non-siliconised plate and fixed in 10% (v/v) glacial acetic acid (BDH) for 30 minutes. The gel was transferred to Whatman N^o3 cartridge paper and vacuum dried at 80 C for approximately 2.5 hours using an Atto dryer and Aquavac pump. The dried gel was exposed to X-ray film (Hyperfilm MP, Amersham UK) for between 24 hours and 1 week depending on the activity date of the [α -³⁵S]d ATP used in the labelling reaction. The DNA sequence was then read directly from the autoradiograph over a light box.

2.7.11 Large scale preparation of plasmid DNA

A large scale preparation of plasmid DNA from the recombinant clones of pT3/T7 harbouring the gB 149bp insert of both wild type and control sequence were prepared using the Qiagen Maxi prep kit (Qiagen Inc. Surrey UK) according to the manufacturer's instructions. The type of purification column used in this case was Qiagen-tip 500 which is appropriate for high copy number plasmids such as pBluescript, pUC, pTZ and pGem with a typical yield of 300-500 μ g DNA from 100ml bacterial culture grown in LB.

A 5ml overnight culture of the two sequenced plasmid clones containing 50 μ g/ml ampicillin in LB was used to sub-culture 100ml of LB supplemented with 50 μ g/ml ampicillin and grown at 37^oC in a shaking incubator (250 rpm) overnight. The bacterial cells were harvested by centrifugation at 6,000rpm in a IECPR-7000 centrifuge for 15 minutes at 4^oC. The bacterial pellet was completely resuspended in 10ml buffer P1 cell resuspension buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 100 μ g/ml RNase A), 10ml

buffer P2 cell lysis buffer (200mM NaOH, 1% SDS) was added and the lysate mixed gently and incubated at room temperature for 5 minutes. 10ml of chilled buffer P3 (neutralisation buffer; 3.0M potassium acetate pH 5.5) was added, the lysate mixed gently but immediately and incubated on ice for 20 minutes in 50ml Falcon tubes. The cell lysate was centrifuged at 20,000g for 30 minutes at 4°C and the supernatant re-centrifuged for a further 10 minutes to ensure removal of all particulate material. Meanwhile a Qiagen-tip 500 for each DNA preparation was equilibrated with 10ml equilibration buffer QBT (750mM NaCl, 50mM MOPS, pH7.0, 15% (v/v) isopropanol, 15% (v/v) Triton X-100) by allowing the buffer to flow through the column by gravity. The cleared supernatant was placed onto the column and allowed to enter the resin by gravity flow, the flow through was discarded and the column washed twice with 30ml QC wash buffer (1.0M NaCl, 50mM MOPS, pH 7.0, 15% (v/v) isopropanol). DNA was eluted off the column by the addition of 15ml QF elution buffer (1.25M NaCl, 50mM Tris-HCl, pH 8.5, 15% (v/v) isopropanol), precipitated with 0.7 volumes (10.5ml) 95% isopropanol at room temperature and centrifuged at 15,000g 4°C for 15 minutes. The DNA pellet was washed twice in 70% (v/v) ethanol, the pellet allowed to air dry and then resuspended in 500µl SDW. The concentration of the DNA was determined by optical density at 260nm where an OD₂₆₀ 1.0 was equivalent to 50µg of double stranded DNA. The stock DNA was stored at -70°C until use.

2.7.12 Linearisation of pT3/T7 gB prior to transcription

The recombinant vectors were linearised initially with *Afl III* prior to the transcription reaction to produce sense (+) RNA of discrete length. 5µg of each of the plasmids was digested with 1µl *Afl III* (10U/µl) in 3µl 10X buffer H (50mM Tris-HCl, 10mM MgCl₂, 100mM NaCl, 1mM DTT pH 7.5) for 3 hours at 37°C. 2µl of the uncut and linearised plasmids was added to 8µl of water and 5µl loading dye and run on a 1% agarose gel together with DNA markers at 75 volts for approximately 30 minutes to verify complete linearisation of the recombinant vectors.

2.7.13 *In vitro* transcription using T3 RNA polymerase and removal of the DNA template

The linearised DNA template was used for *in vitro* transcription using the RiboMax Large Scale RNA production System (Promega) to make sense (+) RNA transcripts. 750ng of the linearised vector DNA with either wild type or control inserted gB sequence was used in the transcription reaction together with 20µl 10X T3 transcription buffer (400mM HEPES-KOH, pH7.5, 120mM MgCl₂, 10mM spermidine, 200mM DTT), 30µl rNTP's (25mM

each ATP, CTP, GTP, UTP), 10 μ l T3 enzyme mix (300u/ μ l T3 RNA polymerase, 15u/ μ l RNasin ribonuclease inhibitor, 190u/ml yeast inorganic pyrophosphate) made up to a final volume of 100 μ l with nuclease free water. The reaction was incubated in a water bath for 3 hours at 37 $^{\circ}$ C and stored at -70 $^{\circ}$ C until use.

2.7.14 Removal of DNase 1 and purification of the RNA transcript

The DNA vector template was removed from the RNA transcription reaction by digestion with RNase free DNase1. 50 μ l of the transcription reaction was treated with 2 μ l RNase free DNase 1 (2u/ μ l Ambion,USA) for 15 minutes at 37 $^{\circ}$ C made up to a total volume of 100 μ l with nuclease free water. The resulting RNA preparation was purified using the RNeasy kit from Qiagen according to the RNA clean up protocol. Briefly, 350 μ l of lysis buffer RLT (10 μ l of β -ME was added to 1ml RLT buffer prior to use), was added to 100 μ l of the RNA solution followed by 100 μ l of 100% ethanol and loaded on to the RNeasy spin column inserted into a clean 2ml ependorf tube and centrifuged in a bench top centrifuge at 8000g for 15 seconds. The flow through was discarded and 500 μ l of column wash buffer RPE (4 volumes 100%of ethanol was added prior to use) was added and centrifuged at 8000g for a further 15 seconds. The column was washed again as above except that the centrifugation step was extended to 2 minutes to dry the membrane. Finally the RNA was eluted from the column with 50 μ l of nuclease free water by centrifugation at 10,000g for 30 seconds and stored at -70 $^{\circ}$ C until use.

2.7.15 Methyl-mercury gel analysis of RNA transcripts

5 μ l of the purified DNase treated purified RNA was analysed on a 2% methyl mercury gel together with non-DNase treated RNA and an RNA molecular weight marker (1000-100 nucleotides, Promega). 1g of low melting point agarose (molecular biology grade VII, Sigma) was added to 50ml 1X EM buffer (50mM boric acid, 5mM Na₂B₄ O₇.10H₂O, 10mM Na₂SO₄, Sigma, adjusted to pH 8.1) and melted in a 750W microwave on full power for approximately 2 minutes. The gel was allowed to cool to 55 $^{\circ}$ C (hand hot) and 250 μ l of methyl mercury hydroxide (1M) was added and mixed. The gel was poured into a clean gel tray containing a 5 well comb which had been previously treated with "RnaZap" and thoroughly washed with DEPC (diethyl pyrocarbonate) treated water to remove any contaminating RNases. 5 μ l of the RNA to be analysed was added to 5 μ l of 2X EM loading dye (25 μ l 1M methylmercuric hydroxide, 500 μ l 4X EM buffer, 100 μ l 100% glycerol, 275 μ l H₂O, 0.2%w/v bromophenol blue). The gel was run in 1X EM buffer at 30-40 volts for approximately 4 hours in a fume hood and stained with ethidium bromide (8 μ l of 10mg/ml in 10ml 1X EM buffer) solution for 1 hour. The gel was visualised with a UV

transilluminator and photographed.

2.8 Optimisation of QC-RT-PCR using control RNA templates

Prior to reverse transcription of the RNA templates, the RNA was checked for contaminating DNA. The pure RNA was diluted to give a range of copies of RNA i.e 10^5 , 10^4 , 10^3 , 10^2 copies for both the wild type and control sequence as determined by optical density, where an $O.D_{260nm}$ of 1.0 was equivalent to $40\mu\text{g/ml}$ RNA. The RNA was amplified by DNA PCR using primers gB1 and gB2 as described for the qualitative PCR in section 2.3.

2.8.1 Reverse transcriptase PCR (RT-PCR) of gB wild type and control sequence

The RNA was titrated to establish the sensitivity of the RT-PCR using the RT-PCR Access system (Promega, USA). Initially the RNA templates were diluted to give 10^5 , 10^4 , 10^3 , 10^2 copies for both the wild type and control sequence. The cycling conditions of the RT-PCR assay were 48°C for 1 minute (1cycle), 94°C for 2 minutes (1cycle), 94°C 30 seconds, 60°C 1 minute, 68°C 2 minutes (40cycles), and a final extension at 68°C for 7 minutes (1cycle). The reaction conditions as recommended by the manufacturer were used for the RT-PCR. The reaction mix was prepared by combining $10\mu\text{l}$ 10 X AMV/Tfl reaction buffer, $2\mu\text{l}$ MgSO_4 (25mM), $1\mu\text{l}$ dNTP mix (10mM each dNTP), 60pmole each of gB1 and gB2 primers, $1\mu\text{l}$ AMV reverse transcriptase ($5\text{u}/\mu\text{l}$), $1\mu\text{l}$ Tfl DNA polymerase ($5\text{u}/\mu\text{l}$) made up to a final volume of $48\mu\text{l}$ with nuclease free water on ice. The positive control DNA reaction components were identical except that the upstream and downstream primers were provided in the kit to amplify a 323bp fragment. For the negative controls every fifth tube, $2\mu\text{l}$ of nuclease free water was substituted for $2\mu\text{l}$ RNA as template. All reaction components were prepared on ice in a separate clean PCR room and the RNA templates were added in a separate PCR set up area taking care to avoid contamination.

2.8.2 Primer and magnesium titration for QCRT-PCR assay

The primers were titrated between a range of 60 pmoles and 10pmoles against 10^3 copies of RNA template. The magnesium was titrated between a range of 0.5-3.0 mM magnesium sulphate against 10^3 copies of RNA template.

2.8.3 Generation of a calibration curve for QC-RT-PCR assay

The wild type and control RNA were titrated against each other to determine the dynamic

range of the QC RT-PCR reaction. The calibration curve was generated by co-amplifying a fixed copy number of the internal control sequence i.e 10^5 copies with a range of copy numbers of the wild type target sequence without the *Hpa 1* restriction site i.e from 10^1 - 10^6 in half \log_{10} steps. The same set of primers and cycling conditions as used for the qualitative RT-PCR assay were used for this purpose except that $2\mu\text{l}$ labelled gB2 from section 2.4.3 was included into the reaction mix to allow for quantitation of the products. As for the quantitative DNA PCR for the gB, following RT-PCR the products were digested with *Hpa1* run on a 12% PAGE gel and exposed to autoradiography film to allow quantitation (section 2.4.4).

2.8.4 Time course of DNA and RNA levels in AD169 infected HEL cells

To assess the comparative levels of DNA and RNA in tissue culture a time course experiment using different multiplicities of infection (moi) of AD169 in HEL cells at time points 0, 6, 12, 24, 48, 72 and 96 hours was set up. 35mm tissue dishes were seeded with 5×10^5 HEL cells in 2ml growth media MEM (Gibco) supplemented with 10% FBS, pen/strep (100U and 10000U respectively). Duplicate dishes for RNA and DNA extraction were grown for 24 hours at 37°C in 5% CO_2 or until 80% confluent. A range of moi's of 0.01, 0.1, 0.5 of AD169 were prepared by dilution of the virus in maintenance media. The media was removed from the cells and the cell sheet washed once with maintenance media. $100\mu\text{l}$ of the appropriate virus dilution was added to the cells and incubated at 37°C in 5% CO_2 for 1 hour. The viral inoculum was removed and the cell sheet washed once with maintenance media, replaced with 2ml maintenance media and incubated for the appropriate time period. The cells were harvested for each time point and both DNA and RNA was extracted.

2.8.5 Extraction of genomic DNA from HEL cells infected with AD169

DNA was extracted from the HEL cells using a Puregene extraction kit (Flowgen Instruments Ltd, UK) according to the manufacturers instructions. The cell monolayer was lysed directly on the surface of the 35mm dish by removing the media and adding $600\mu\text{l}$ cell lysis solution with vigorous pipetting to ensure complete lysis of the cells. The lysate was added to a 1.5 ml ependorf tube, $3\mu\text{l}$ RNase solution was added, the tube inverted several times and incubated at 37°C for 15 minutes to remove the RNA. After cooling to room temperature, $200\mu\text{l}$ of protein precipitation solution was added, vortexed vigorously and centrifuged at 13,000g for 3 minutes to form a tight white pellet of protein. The supernatant containing the DNA was precipitated with $600\mu\text{l}$ 100% isopropanol in a new 1.5ml tube by inverting the tube until the DNA was visible and centrifuged at 13,000g

for 1 minute. The DNA pellet was washed twice with 600 μ l 70% ethanol, resuspended in 50 μ l of SDW and stored at -70°C until use.

2.8.6 Extraction of RNA from HEL cells infected with AD169

RNA was extracted from the cells using the commercially available RNeasy kit (Qiagen Ltd, UK). The media was removed from the cells, 350 μ l of lysis buffer RLT (10 μ l of β -ME per 1ml lysis buffer added prior to use) was added directly to the monolayer and pipetted vigorously. The cell lysate was added directly onto a QIAshredder column (Qiagen Ltd, UK) and centrifuged for 1 minute at 13,000g. One volume of 70% ethanol (350 μ l) was added and the tube inverted several times, the mixture was applied to an RNeasy column and centrifuged at 8,000g for 15 seconds. The eluate was discarded and the column containing the bound RNA placed into a clean 2ml tube. The column was washed once with 700 μ l wash buffer RW1 and then washed twice with 500 μ l wash buffer RPE by centrifuging at 8,000g for 15 seconds and removing the eluate and finally for 2 minutes to completely dry the column. The RNA was eluted from the column by adding 25 μ l nuclease free water to the column and centrifuging at 8000g for 1 minute, the RNA was stored at -70°C until use.

2.9 HCMV Disease Definitions

HCMV disease was diagnosed when typical features occurred within two weeks of the detection of HCMV infection as described at the fifth International CMV Workshop, Sweden 1995 (Ljungman and Plotkin).

Debilitating febrile illness - fever spiking to 38°C or above for at least 48 hours in the absence of rejection or bacterial/fungal infection, with neutropenia, in association with HCMV viraemia.

Hepatitis - alteration in liver function tests (AST > twice upper limit of normal) on at least two consecutive days in absence of bacterial or fungal infection, in association with HCMV shedding from any site.

Pneumonitis - chest symptoms and/or a characteristic chest X-ray pattern unresponsive to antibiotics and with evidence of HCMV infection in bronchoalveolar lavage fluid.

DNAemia - for the purpose of this thesis is defined as the detection of HCMV DNA in

blood.

Viraemia - is the detection of HCMV DNA in whole blood by qualitative PCR.

Viral load - is the quantity of HCMV in either blood or urine expressed as genomes/ml.

2.10 Statistical Methods

The significance of differences between maximum viral loads in symptomatic and asymptomatic patients, D+R-, D+R+ and D-R+ groups or patients with or without viraemia were assessed by the Mann-Whitney U test (Altman, 1993). Initially, univariate relationships were studied by drawing up two by two contingency tables of HCMV disease (yes/no) against either viraemia (yes/no) or donor serostatus (positive/negative). The relationships between these variables were then tested for significance using chi-squared test (viraemia) or Fisher's exact test (donor status). The relationship between two continuous variables from the same individuals was analysed with Spearman's rank correlation coefficient. Viral load differences between those with and without HCMV disease were studied using the Mann-Whitney U test. Where significant, these relationships were then quantified using univariate and multivariate logistic regression analysis (Altman, 1993). For these analyses, the odds ratios quoted refer to each 0.25 \log_{10} increase in viral load or each 1g increase in methylprednisolone. The results from this data set were used to generate an equation to determine the expected probability of being symptomatic at any given viral load. Predicted probabilities from the final logistic models were plotted against viral load. All analyses were carried out using PRO LOGIST in the statistical analysis system (SAS, 1989).

Chapter 3.

Modulations in cytomegaloviral load in the urine of HCMV congenitally infected infants and the effects of antiviral therapy

3.1 Introduction

Human cytomegalovirus is the single most important virus responsible for congenitally acquired infections with an incidence of between 0.3 - 1.2% of all live births in the U.S.A per year (Stagno *et al* , 1980, 1982, 1986, Alford *et al*, 1991, Hanshaw *et al*, 1995). The virus can be transmitted to the fetus as a congenital infection in utero or as a perinatal infection via the genital tract during delivery or by breast feeding. There are several factors which can influence the clinical outcome of infected infants including; the mode of transmission (perinatal or congenital), the immune status of the mother and socioeconomic conditions. Stagno *et al* (1982) in the largest study of its kind, highlighted the importance of maternal serostatus with respect to risk of transmission to the fetus by comparing the relative importance of primary and recurrent infection in 3712 pregnant women. This, together with many subsequent studies showed the greatest risk of transmission to the infant was acquisition of virus from a primary maternal infection during pregnancy with an incidence of between 35% and 37% (Fowler *et al*, 1992, Donner *et al*, 1993, Bopanna, *et al* , 1995, Liptz *et al* , 1997). Recurrent maternal infection found predominantly in lower socioeconomic groups is associated with considerably lower rates of transmission and results in mainly asymptomatic infections. This lower risk to the infant is presumably due to the transfer of protective maternal antibodies (Stagno *et al*, 1982, Fowler *et al* , 1992, Bopanna *et al*, 1993, 1995, 1996b).

More recent studies have confirmed the importance of protective immunity provided by maternal seropositivity in congenital infection (Tankara *et al*, 1991, Fowler *et al* 1992, Hirota *et al*, 1992). Tankara *et al* (1991) showed an increase in neutralising antibody titres during pregnancy from the first to the third trimester in 82 pregnant women with prior immunity to HCMV. Since these antibodies are able to cross the placenta, they may have an important role in protecting the fetus from intrauterine infection resulting in a more favourable clinical outcome for the infant. However, there are confounding reports as to the relevance of the detection of neutralising antibodies as they may reflect the extent of virus replication. Indeed, Bopanna *et al* (1996b) showed that increased titres of neutralising antibodies to gB were observed in infants that developed sequelae. Fowler *et al* (1992) showed symptoms at birth only in infants from mothers who underwent a primary infection during pregnancy, 25% of whom developed more than one sequelae upon follow up at 4.7 years compared to only 8% of infants from mothers experiencing recurrent infection. There have been reports of symptomatic congenital infection in pre-immune mothers albeit rarely as isolated case reports (Ahlfors *et al*, 1981, Rutter *et al*, 1985, Morris *et al*, 1994).

The route of perinatal infection is very difficult to establish due to the numerous bodily secretions that harbour the virus. However, the genital tract during birth and breast milk have both been identified as major routes of transmission (Stagno *et al*, 1980, Alford *et al*, 1991). Acquisition of the virus via the genital tract results in approximately 50% of the infants becoming infected compared to 40% during the first month of breast feeding (Alford *et al*, 1991). Although the risk of infection is similar between the two modes of transmission, there is a considerably less favourable outcome for infants infected via primary maternal infection in utero within the first trimester. The clinical manifestations of symptomatic infants at birth are predominately microcephaly, hepatosplenomegaly, jaundice and petechial rash often associated with raised ALT levels and thrombocytopenia. Of those infants born with symptomatic infection, between 80-90% develop severe sequelae including mental retardation, sensorineural hearing loss seizures and chorioretinitis, whereas only 8-15% of babies born with congenital HCMV without symptoms at birth develop severe sequelae later on.

The most challenging problem to control congenital HCMV infection are diagnosis and treatment. The "gold standard " method for diagnosis of congenital HCMV is by virus isolation within the first three weeks of life from either urine or saliva. The main disadvantage of this diagnostic test is that it may take up to three weeks before cpe appears. To date, several virological methods have been used to identify congenital infection with different degrees of success. Prenatal diagnosis may be achieved by testing amniotic fluid by PCR while serological testing in this context is of little use due to the poor sensitivity and specificity of IgM tests currently available (Donner *et al*, 1993, Ruellan-Eugene *et al*, 1996, Lipitz *et al*, 1997). Several studies have attempted to provide prognostic information using PCR to detect HCMV DNA in a variety of clinical samples including sera, urine, amniotic fluid and peripheral blood leukocytes (Donner *et al*, 1993, Hogge *et al*, 1993, Troendle-Atkins *et al*, 1994, Darin *et al*, 1994, Revello *et al*, 1995, Nelson *et al*, 1995, Ruellan-Eugene *et al*, 1996). The most promising results for prenatal diagnosis of HCMV infection have been obtained by detection of DNA in the amniotic fluid (Donner *et al*, 1993, Hogge *et al*, 1993, Revello *et al*, 1995, Lipitz *et al*, 1997). Indeed, Revello *et al* (1995) reported a positive predictive value of 100% and a negative predictive value of 81% in their study, while Donner *et al* identified 13/16 cases of HCMV infection using PCR . However, the timing of amniocentesis or acquisition of the virus and type of maternal infection can all influence the reliability of the assay. In another study by Nelson *et al* (1995) serum PCR was used to identify 18/18 symptomatic congenital infections at birth which provides encouraging results for the

application of a less invasive technique to diagnose HCMV infection in congenitally infected infants.

The association between the quantity of HCMV virus in the urine of congenitally infected infants and disease was first established by Stagno and colleagues in 1975 (Stagno *et al*, 1975). The results from this pioneering study showed a significant relationship between clinically overt disease and increased virus load in the urine at birth of congenitally infected infants versus asymptomatic natally infected infants. More recent studies using modern molecular biological techniques have also found a direct relationship between elevated virus load and disease in a variety of immunocompromised patient groups (Fox *et al*, 1995, Drouet *et al*, 1995, Bowen *et al*, 1995 & 1996). To date however, no such detailed quantitative analysis using these more sensitive molecular techniques have been performed for the long term follow up of congenitally infected infants with HCMV.

Treatment of congenital HCMV is not usually undertaken due to the toxicity of the currently available antiviral compounds. However, one study evaluating the use of ganciclovir in a phase/II clinical trial for symptomatic congenital HCMV infection with a three year follow up demonstrated a hearing improvement in 16% of the 30 babies enrolled (Whitley *et al*, 1997). However, more extensive controlled trials with long term follow-up are required before any treatment protocols based on ganciclovir therapy can be recommended for treatment of congenital HCMV. There have been no studies to date in the congenital population to investigate the safer but probably less potent antiviral compound aciclovir.

This chapter describes data obtained using both qualitative and quantitative PCR assays to examine the modulations in HCMV load in the urine of congenitally infected infants over time. Longitudinal load measurements were also used to examine the effect of antiviral therapy on HCMV viral load and estimated viral clearance in HCMV infected infants. Although this study was not a randomised controlled trial, the aim was to make a comparison between infants receiving ganciclovir and non-treated infants in an attempt to provide evidence for reduced viral shedding. The data obtained from the examination of virus load and the effects of antiviral therapy may provide important information into the natural history of congenital infection and assist in the development of future treatment regimens.

3.2 Patients studied

3.2.1 Study Population

Urine samples from twenty suspected cases of congenital HCMV between 1993 and 1995 were collected and followed prospectively for evidence of HCMV infection initially using a qualitative PCR assay. Eleven of the cases were recruited from Graz, Austria (patients 7,8 and 12-20), while the remaining nine cases presented at or were referred to the Royal Free Hospital, London (patients 1-7, 10,11). The first urine sample collected within the first three weeks of life was subjected to virus isolation to confirm congenital infection. Subsequently, urine samples were tested for HCMV DNA using qualitative PCR without processing at both neat and a 1:20 dilution. All urine samples qualitatively PCR positive at either dilution were then tested using the quantitative PCR assay for HCMV DNA as described in chapter 2 section 2.4. In addition if available, the first urine sample from each patient who presented at the Royal Free Hospital was also subjected to TCID₅₀ assay in human embryo lung cells (described in chapter 2 section 2.5.4). Unprocessed urine and CSF samples received from Austria, were subjected to the same qualitative and quantitative PCR assays for HCMV DNA.

3.2.2 Antiviral therapy

Two infants were administered aciclovir therapy (30mg qid), one infant was treated for forty two days (patient 10), the other infant received aciclovir therapy for 50 days (patient 11). Nine of the eleven infants from Austria were administered ganciclovir therapy (10mg/kg iv) for three weeks, where therapy was initiated following the first PCR positive result in urine. Two of the infants receiving ganciclovir therapy experienced suspected toxicity. One infant suffered leukopenia and one had bloody diarrhoea, as a result the dose was reduced to 5mg/kg for the remaining two weeks. None of the remaining nine infants received any antiviral therapy.

3.2.3 Diagnosis of Congenital HCMV

Congenital HCMV diagnosis was based upon the isolation of HCMV from either urine or saliva within the first three weeks of life in all twenty cases. In addition any other clinical or laboratory indications of congenital infection were also reported for each infant and are detailed in Table 3.1.

3.3 Results

3.3.1 Patients

Table 3.1 shows the baseline demographics of the 20 infants with congenital HCMV infection, detailing age at the time of initiation of PCR surveillance, sex, antiviral therapy, HCMV TCID₅₀ result where available, any clinical parameters during surveillance and the HCMV viral load from the first available urine sample. The median follow up of the twenty suspected cases of congenital HCMV was 185 days post-partum (range, 30 - 719 days post-partum) and the median age of the infants at the start of the analysis was 8 days (range 1 - 92 days). The age of the infants at baseline and time of follow up was comparable between the eleven infants receiving antiviral therapy (median age=6 days, range 2 - 27; median follow up =303 days; range, 30 - 461 days) and the nine non-treated infants (median age=28 days, range 1 - 92 ; median follow up=142 days, range, 91 - 337 days; p>0.05 Mann-Whitney test).

3.3.2 Qualitative PCR results

HCMV DNA was detected by PCR in the urine of all twenty infants with congenital HCMV infection indicating active viral replication. A total of 112 urine samples were initially subjected to qualitative PCR (median=5 per patient, range 3 - 12) of which 94 samples were PCR positive. The remaining 18 PCR negative samples were from those patients receiving ganciclovir therapy. The median number of urine samples analysed from infants receiving antiviral therapy was significantly higher (median= 7; range 3 - 12) than the non-treated infants (median=4; range 3 - 8; p<0.01, Mann-Whitney U test). The nine infants not receiving antiviral therapy and the two infants administered low dose aciclovir had at least three or more urine samples which were consecutively PCR positive. In contrast, six out of nine infants (66%) administered ganciclovir became PCR negative in urine within the first month of treatment while three (33%) remained consistently PCR positive during and after therapy. Of the 11 infants administered ganciclovir therapy, 5 (45%) were also PCR positive in CSF at presentation but asymptomatic at birth, while 6 (55%) were PCR negative, two of whom were symptomatic at birth.

3.3.3 Quantitative PCR results

All urine samples that were qualitatively PCR positive during the surveillance period were subjected to HCMV quantitation. The median viral load from the 94 PCR positive urine samples was $10^{5.98}$ log₁₀ genomes/ml urine (range $10^{3.30}$ - $10^{9.07}$ log₁₀). Prior to antiviral therapy the median viral load was $10^{7.0}$ log₁₀ genomes/ml urine (n=11; range $10^{3.30}$ - $10^{9.03}$)

which was comparable to the baseline HCMV viral load in the remaining nine infants not receiving therapy (median= $10^{7.77}$ log₁₀ genomes/ml ;range $10^{4.81}$ - $10^{8.36}$; $p>0.05$). When considering analysis of all urine samples subjected to HCMV quantitation, patients receiving antiviral therapy had a significantly lower median viral load of $10^{4.77}$ log₁₀ genomes/ml (n=73, range 0 - $10^{9.02}$ log₁₀) than the non-treated infants (median= $10^{6.95}$ log₁₀ genomes/ml, range $10^{4.19}$ - $10^{9.07}$; $p<0.0001$ Mann-Whitney test) despite comparable follow up times between each group. Indicating a prolonged effect of antiviral therapy in reducing HCMV replication. In order to assess the effect of antiviral therapy on virus load in infants receiving either ganciclovir or aciclovir compared to those infants without any antiviral intervention, the total area under the viral load time curve was calculated and compared between the two groups. There was a significant difference when comparing total area under the curve of viral load calculated per day between the eleven treated (median= 3.77 log₁₀ genomes/ml urine/day; range 0.264 - 6.92 log₁₀ genomes/ml urine/day) and the nine non-treated infants (median= 6.08 log₁₀ genomes/ml urine/day; range 5.19 - 7.94log₁₀ genomes/ml urine/day : $p<0.01$, Mann-Whitney test). These data are illustrated in figure 3.1 where the dotted line indicates the median viral load for all samples that were quantitated.

3.3.4 Modulations in HCMV viral load in infants not receiving antiviral therapy

The longitudinal viral load fluctuations for infants not receiving antiviral therapy are shown in figure 3.2. HCMV could be detected continuously during follow up in all nine infants. The profiles show that fluctuations in HCMV load occur over time, with HCMV still being detected at late times following presentation. Indeed, at no time did the HCMV viral load in urine fall below $10^{4.0}$ log₁₀ genomes/ml urine. The median HCMV viral load for the first sample was $10^{7.77}$ log₁₀ genomes/ml (range $10^{4.81}$ - $10^{8.36}$) which was not significantly different to the median viral load in the final urine sample at a median of 303 days follow up (median= $10^{6.09}$ log₁₀ genomes/ml , range $10^{5.48}$ - $10^{8.33}$: $p>0.05$, Mann - Whitney test) indicating constant shedding of virus at high levels even up to 337 days post-partum (patient 7).

Table 3.1 Baseline demographics of all 20 congenitally infected infants in the study detailing HCMV viral loads prior to therapy. +ve (positive), -ve (negative), M (male), F (female), PP (post-partum), N/A (not available), IUGR (intrauterine growth retardation), D&V (diarrhoea and vomiting), LET (liver function test), CSF (cerebrospinal fluid), PCP (*pneumocystis carinii*).

Patient	Age	Sex	Antiviral	HCMV log ₁₀ genomes/ml	TCID ₅₀	CSF	Clinical details
1	92	F	None	8.04	N/A	N/A	Premature, chronic lung disease
2	28	F	None	4.95	1 in 67	+ve	Premature, pneumonitis (67 days PP)
3	46	F	None	6.42	N/A	N/A	IUGR, mild microcephaly, ?pneumonitis, D&V (38 days PP)
4	25	M	None	7.94	N/A	N/A	Pyrexia, raised LFT (25 days PP)
5	89	M	None	7.77	N/A	N/A	Pneumonitis, HIV+ve baby, PCP+ve
6	81	M	None	8.35	1 in 5x10 ⁴	N/A	?
7	3	M	None	7.65	N/A	-ve	CSF ↑ protein, calcification in brain, mental retardation, diplegia, hearing impairment
8	1	M	None	4.81	N/A	+ve	Asymptomatic
9	3	F	None	8.16	N/A	-ve	Asymptomatic
10	24	F	ACV	6.80	1 in 3x10 ³	N/A	?
11	27	M	ACV	6.65	1 in 1x10 ³	N/A	?
12	8	M	GCV	4.62	N/A	+ve	Asymptomatic
13	4	F	GCV	8.07	N/A	+ve	Asymptomatic
14	3	M	GCV	3.30	N/A	N/A	Septic at birth, light mental retardation
15	3	F	GCV	7.26	N/A	-ve	Asymptomatic at birth, diplegia resolved with therapy
16	8	F	GCV*	6.03	N/A	-ve	Asymptomatic at birth, slight hearing impairment on follow up
17	2	M	GCV	7.79	N/A	-ve	Asymptomatic at birth and follow up
18	9	F	GCV	7.07	N/A	+ve	Asymptomatic at birth and follow up CSF ↑ protein
19	6	M	GCV	7.00	N/A	-ve	Hydrocephalus at birth, light mental retardation at follow up
20	4	M	GCV*	9.03	N/A	+ve	Asymptomatic at birth and follow up, CSF ↑ protein

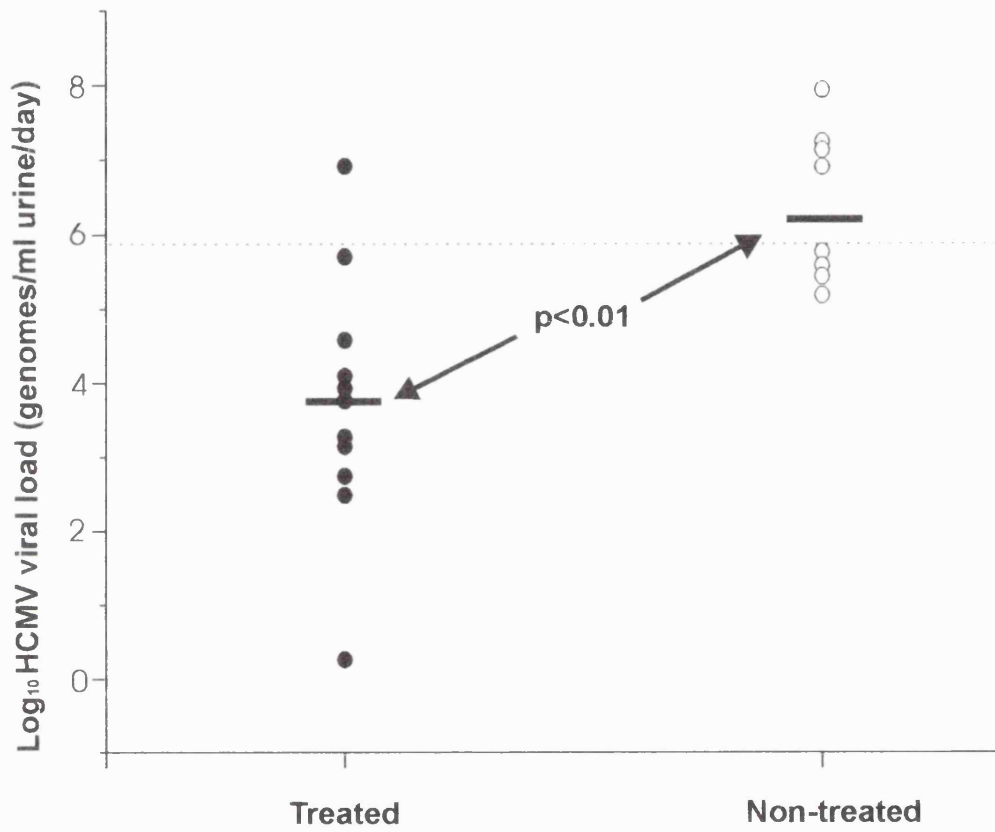


Figure 3.1 Scatter diagram of area under the curve of HCMV viral load per day in the urine of 20 congenitally infected infants. The dotted line indicates the median viral load for all 49 urine samples subjected to quantitation. The solid lines indicate the medians (Mann-Whitney U test).

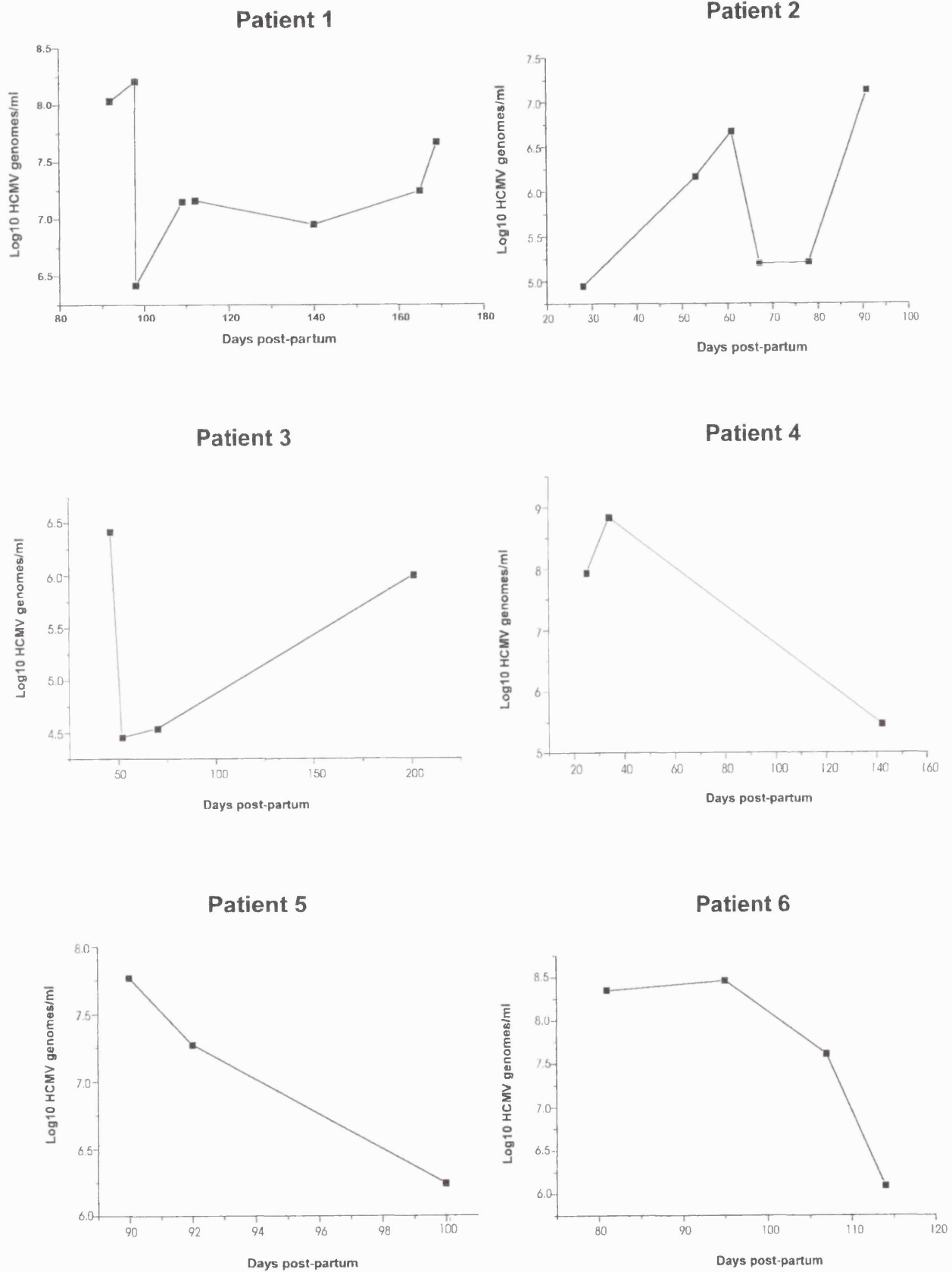
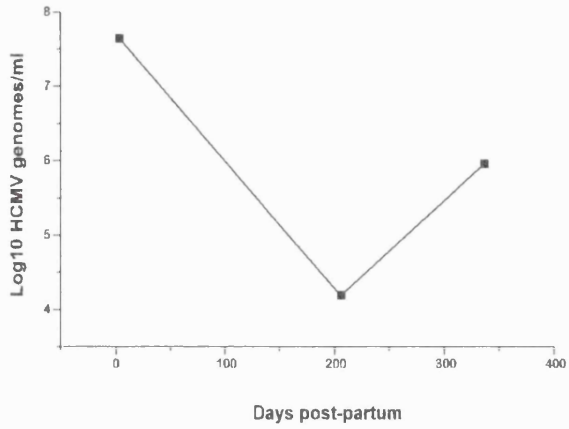
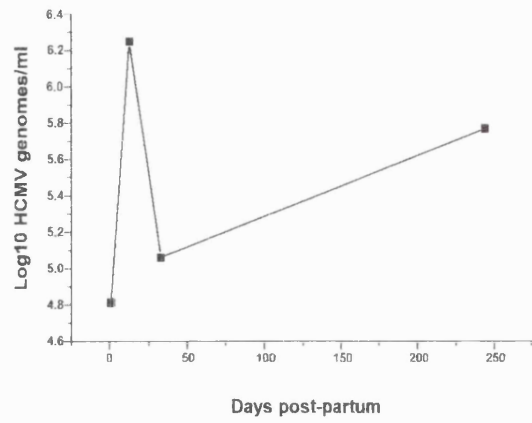


Figure 3.2 Longitudinal HCMV viral load profiles in nine congenitally infected infants not receiving antiviral therapy (note differing scales).

Patient 7



Patient 8



Patient 9

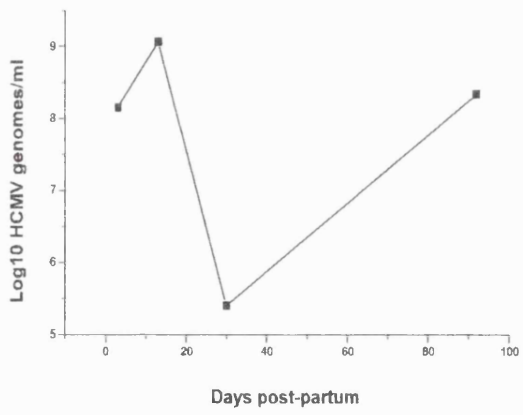


Figure 3.2 Continued (note differing scales).

3.3.5 Modulation in HCMV viral load in infants receiving antiviral therapy

The longitudinal HCMV viral load profiles from those infants receiving antiviral therapy are shown in figure 3.3. In all infants receiving therapy, there was a reduction in HCMV load during treatment which was more sustained in those individuals receiving the more potent antiviral agent ganciclovir. Indeed, in six infants (66%) receiving ganciclovir the viral load was reduced to below detectable levels (approximately $2.30 \log_{10}$ genomes/ml urine or blood). In contrast to the non-treated infants, the viral load in the first urine sample (median= $10^{7.00} \log_{10}$ genomes/ml, range $10^{3.30} - 10^{9.03}$) was significantly different from the last PCR positive follow up sample (median= $10^{5.23} \log_{10}$ genomes/ml, range $10^{2.30} - 10^{6.78}$; $p < 0.05$) despite comparable follow up times between the two groups and a median of 170 days (range 6 - 434 days) after therapy had ceased.

3.3.6 Relationship between HCMV viral load and response to antiviral therapy

The effect of antiviral therapy was analysed further by considering the \log_{10} reduction in viral load with respect to time in the eleven treated infants. Figure 3.4 shows the changes in HCMV viral load during therapy, infants 12-20 received ganciclovir for 3 weeks, while infants 10 and 11 received aciclovir therapy for 42 and 50 days respectively. There was a marked reduction in HCMV viral load in all patients except for patient 10, who during aciclovir therapy had a rebound of virus replication. When the pre-treatment viral load was compared to the first urine sample after therapy had stopped, 5 patients (45%) became PCR negative, corresponding to a mean reduction in viral load of $3.16 \log_{10}$ genomes/ml urine (range $0.75 \log_{10} - 6.73 \log_{10}$). Patients 12 and 14 remained HCMV PCR negative after therapy had stopped, however, the follow up times for these two infants was only 30 and 53 days respectively. In the remaining nine infants, HCMV DNA was still detected as shown in figure 3.5. Three infants (patients 13,15,18) remained PCR negative for at least two months before virus was detected again, while six infants (patients 12, 14,16,17,19 &20) had detectable virus throughout surveillance.

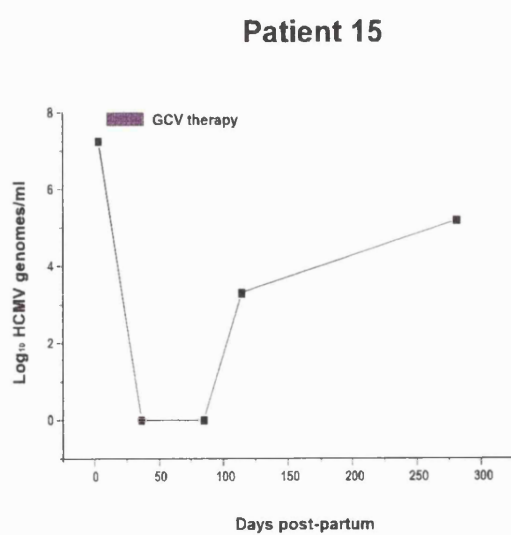
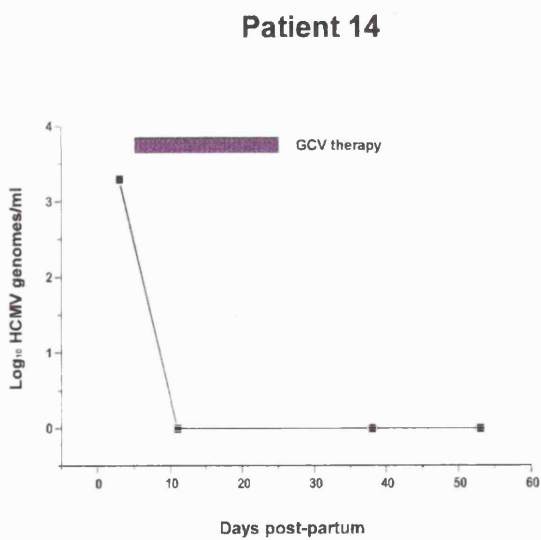
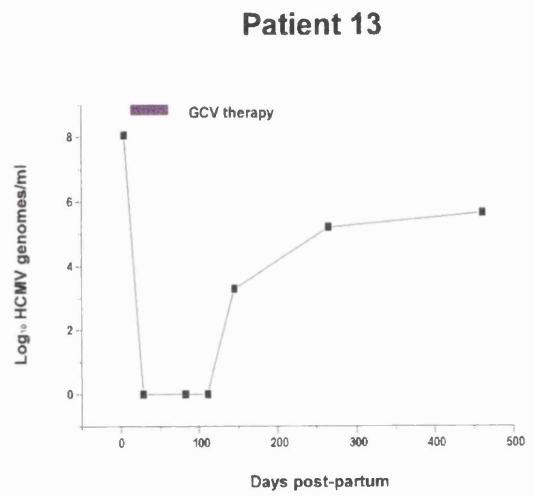
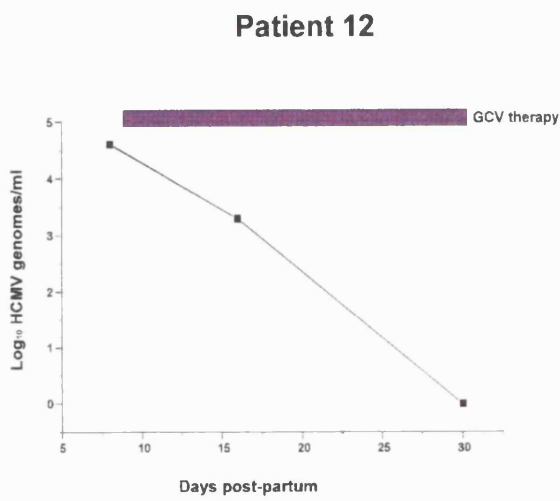
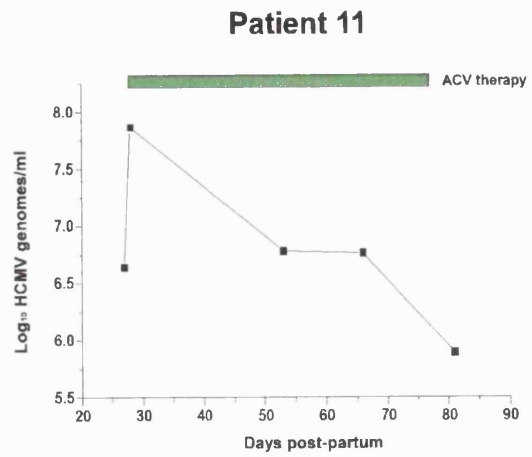
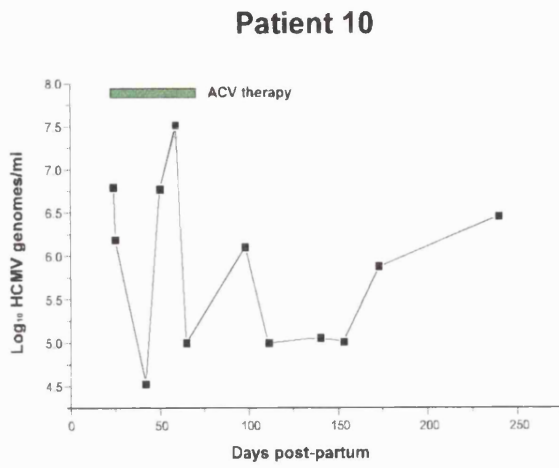
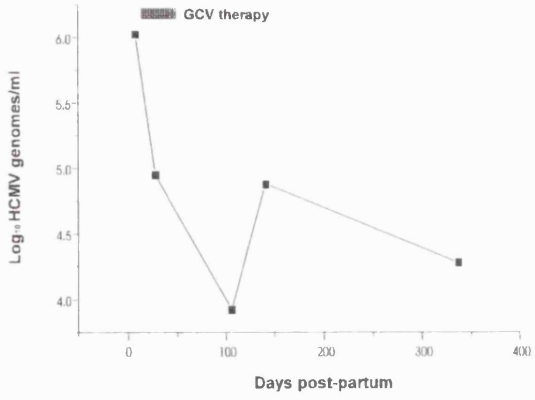
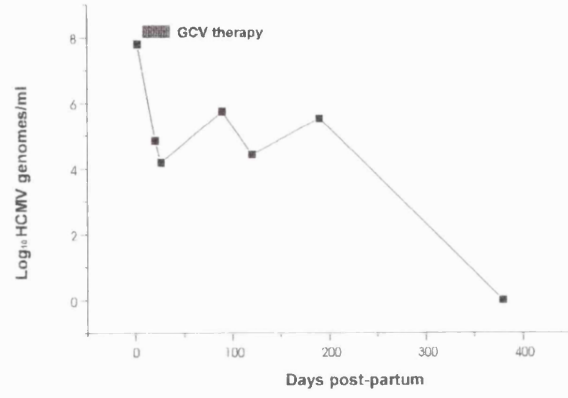


Figure 3.3 Longitudinal HCMV viral load profiles in 11 congenitally infected infants receiving either aciclovir (patients 10 & 11) or ganciclovir (patients 12 - 20), (note differing scales)

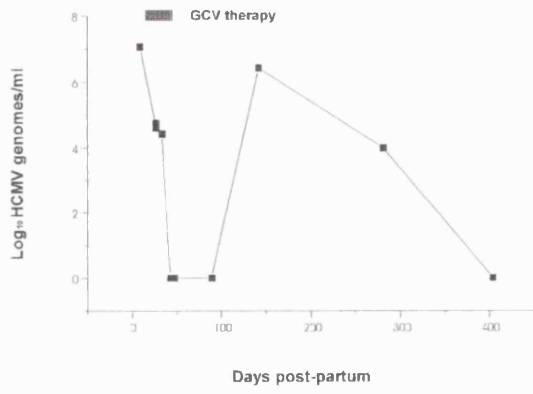
Patient 16



Patient 17



Patient 18



Patient 19



Patient 20

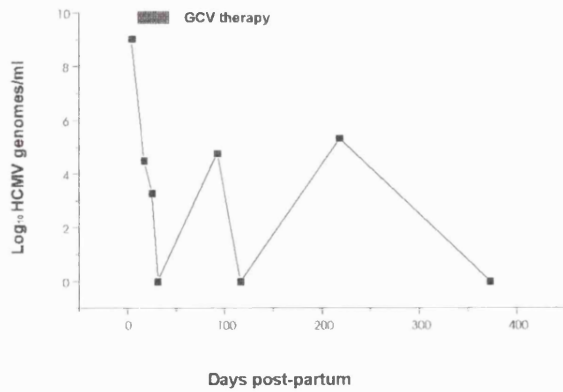


Figure 3.3 Continued (note differing scales).

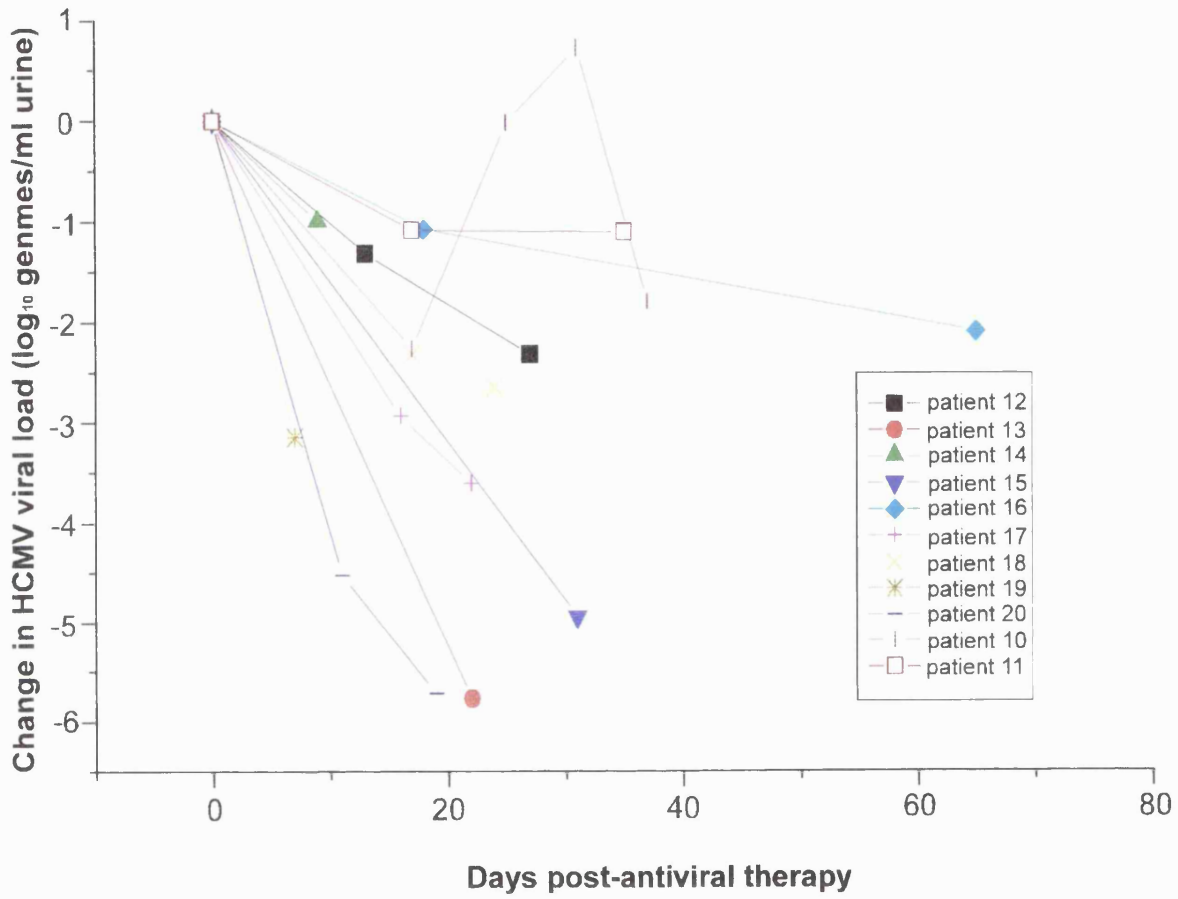


Figure 3.4 Changes in HCMV viral load during antiviral therapy in 11 congenitally infected infants (patients 10 & 11 were administered aciclovir, patients 12 - 20 received ganciclovir).

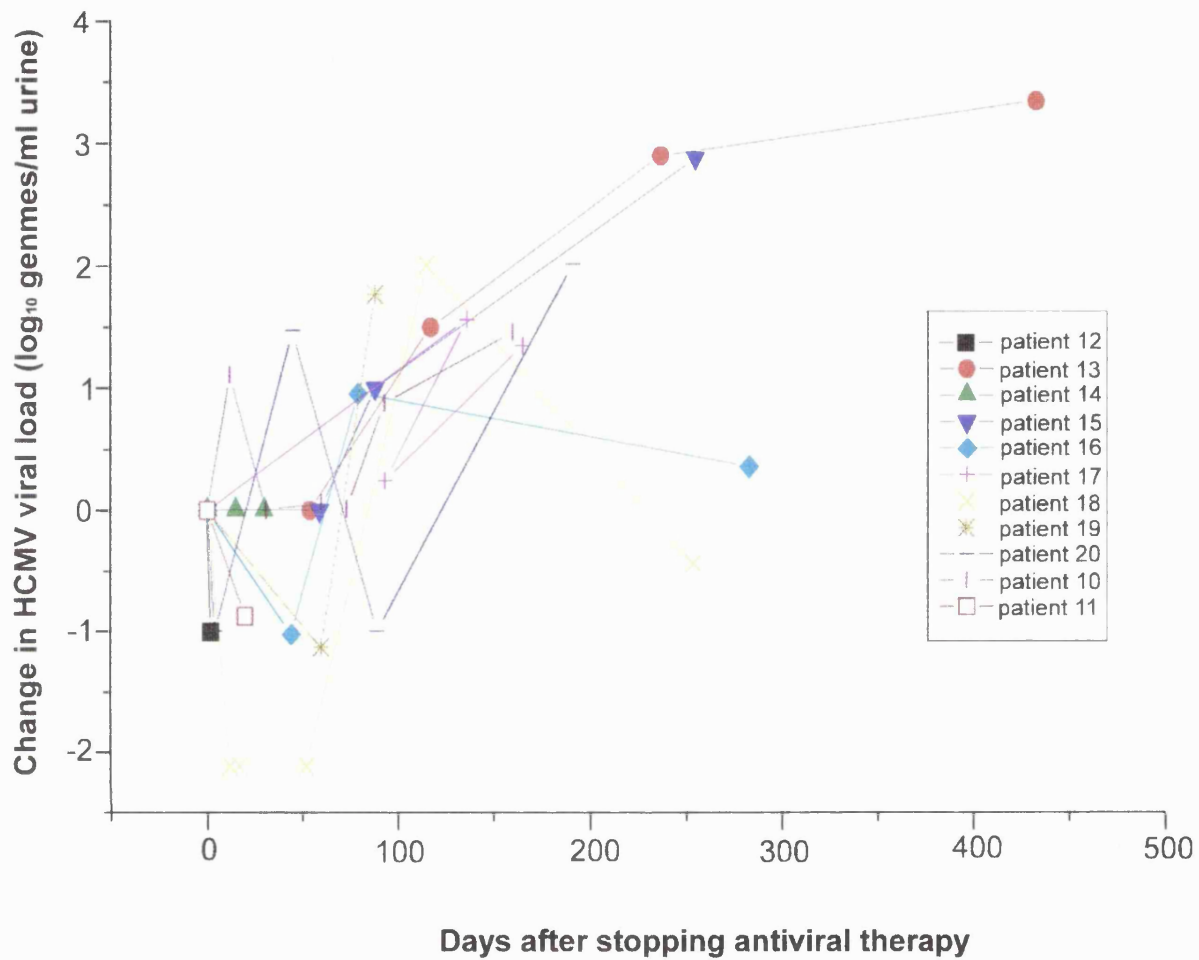


Figure 3.5 Changes in HCMV viral load in 11 congenitally infected infants after antiviral therapy had stopped.

3.3.7 HCMV viral clearance rates in infants receiving antiviral therapy

In order to analyse the effect of antiviral therapy on virus replication, the viral load clearance rate was calculated for those infants receiving treatment. The estimated half life of the virus in urine ($t_{1/2}$) was determined using Equation 1 by calculating the slope of decline for reduction in virus load during therapy with respect to time.

Equation 1. $t_{1/2} = \ln 2 / (\text{slope of decline})$

$$\text{slope of decline} = \frac{\ln [\text{HCMV genomes/ml Day 0}] - \ln [\text{HCMV genomes/ml Day x}]}{t}$$

where Day x = the first PCR result following cessation of therapy and t= time elapsed between Day 0 and Day x

The individual viral clearance rates together with HCMV viral loads prior to therapy and at the first PCR result after therapy had stopped are shown in table 3.2. The pre-treatment HCMV viral load was not related to the $t_{1/2}$ in the 11 infants receiving antiviral therapy. The mean half life for HCMV clearance during ganciclovir was 1.65 days (median=1.44 days, range 0.48 - 4.47). The slowest clearance rate was found in patient 16 who had received a reduced dose of ganciclovir due to toxicity to the drug. However, patient 20 also received a reduced dose but had the fastest clearance rate (0.59 days). Inclusion of the two patients receiving aciclovir therapy increased the median $t_{1/2}$ slightly to 1.83 days (mean= 2.02 days).

Table 3.2 HCMV viral clearance rates ($t_{1/2}$) for 11 infants receiving antiviral therapy, 2 patients received aciclovir therapy (10,11) 9 received ganciclovir therapy (12-20).

Patient	Log₁₀ genomes/ml before therapy	Log₁₀ genomes/ml after therapy stopped	$t_{1/2}$ (days)
10	6.80	5.00	2.90
11	6.65	5.89	4.38
12	4.62	2.30	1.04
13	8.07	2.30	1.04
14	3.30	2.30	2.10
15	7.23	2.30	1.83
16*	6.03	3.92	4.47
17	7.79	4.18	1.44
18	7.07	4.42	1.90
19	7.00	3.88	0.48
20*	9.03	2.30	0.59

* Infants receiving reduced ganciclovir therapy due to suspected toxicity

3.4 Discussion

The aim of this chapter was to investigate the modulations in HCMV viral load in the urine of congenitally infected infants over time. The qualitative and quantitative PCR assays utilised for this purpose provide an insight into the extent of viral replication. Unlike previous studies, this methodology provides a rapid and non-invasive technique to investigate congenital HCMV infection (Donner *et al*, 1993, Hogge *et al*, 1993, Dong *et al*, 1994, Revello *et al*, 1995, Lipitz *et al*, 1997). All twenty cases were confirmed by virus isolation from urine within the first three weeks of life and so fulfill the criteria for diagnosis of congenital HCMV infection. For the nine non-treated infants, HCMV could be detected throughout the surveillance period, in contrast to infants receiving antiviral therapy, where 66% of infants treated with ganciclovir became PCR negative during therapy.

Five infants without symptoms at birth were qualitatively PCR positive for HCMV DNA in CSF, four of these infants received ganciclovir therapy. Troendle - Atkins *et al* (1994) reported similar PCR positivity in CSF samples, thus questioning the reliability of this highly invasive technique for the prognosis of congenital infection. In the study by Troendle - Atkins *et al*, 13 infants with symptomatic HCMV were analysed, only 60% of whom were qualitatively PCR positive for HCMV DNA in CSF prior to antiviral therapy. However, there was a significant correlation between PCR positivity and poor neurodevelopment upon long term follow up in five of the infants. The data suggest that further studies involving larger numbers of infants with long term follow up are required to assess the prognostic value of CSF PCR.

The results presented in this chapter show that HCMV viral load fluctuates considerably in infants not receiving antiviral therapy as illustrated by the longitudinal profiles in figure 3.2, where the viral load ranged between $10^{4.81} \log_{10}$ and $10^{8.36} \log_{10}$ genomes/ml urine but did not fall to below $10^{4.0}$ genomes/ml urine at any time during the surveillance period. The median viral load of the first urine sample was not significantly different to that of the final urine sample in untreated infants despite a median follow up of 142 days. This observation is consistent with the data obtained by TCID₅₀ measurement of urine samples obtained at three monthly intervals by Stagno *et al* (1975). These data show that virus excretion can persist for a long time following congenital infection and does not appear to be influenced by the maturity of the immune system. Stagno *et al* were the first to demonstrate that increased quantity of virus present in urine within the first few weeks of life was significantly associated with poor prognosis. From the group of nine non-

treated infants presented in this chapter, only two had symptoms at birth (patients 3 & 7), patient 7 had a higher viral load than the median observed within the group, whereas patient 3 had a lower viral load than the median at presentation. Whether this reflects the state of immune tolerance to the virus, or the strain and quantity of virus inoculum cannot be answered from these data alone and requires analysis of larger groups of infants with prolonged follow up.

The effect of antiviral therapy on virus load was analysed in those patients receiving either ganciclovir or aciclovir and was compared to infants not receiving any antiviral therapy. The median viral load in infants receiving antiviral therapy was significantly lower than in infants not receiving therapy when considering all samples analysed. This effect was more apparent in infants treated with ganciclovir, where six out of nine infants became PCR negative in urine, while the two infants receiving aciclovir therapy remained PCR positive throughout surveillance. In contrast all of the nine none treated infants remained PCR positive throughout follow up. This observation is consistent with the relative antiviral potency of ganciclovir and aciclovir on HCMV replication. The prolonged effect of antiviral therapy was further demonstrated by the significant difference when comparing the viral load from the first and last urine sample in treated and non-treated infants despite comparable follow up times between the two groups. When the area under the curve per day was compared between the two groups, there was a significantly higher viral load per day in the non-treated infants. Infants receiving antiviral therapy had a significantly lower viral load in the last follow up sample, whereas there was no difference seen for the nine infants not receiving antiviral therapy. Indeed, three infants receiving ganciclovir remained PCR negative for two months after therapy had stopped further demonstrating the sustained antiviral effect on virus replication. These data may have important implications for the prolonged effect of antiviral therapy at reducing viral replication long after therapy had stopped. This may result in reducing severe sequelae such as mental retardation or sensorineural hearing loss in infants with congenital infection that often does not appear until much later on in life.

The access to relatively frequent samples following initiation of potent antiviral therapy allowed the half- life of HCMV clearance in the urine to be estimated. These data provide for the first time in congenitally infected infants an estimate of virus clearance rates in the presence of ganciclovir and aciclovir. In infants receiving ganciclovir the mean clearance rate was 1.65 days. Surprisingly, the lowest and highest clearance rates were found in the two individuals both receiving a reduced dose of ganciclovir due to toxicity, which was

independent of the viral load prior to therapy. This suggests that the ability to respond to ganciclovir therapy is not solely based on the quantity of virus inoculum since both infants had comparable sampling and follow up times.

The results in this chapter clearly show that virus excretion in congenitally infected infants not receiving antiviral therapy fluctuates with time. Whereas infants treated with ganciclovir experienced a dramatic reduction in HCMV viral load to below detectable levels very rapidly. These data are in agreement with a recently published phase II study investigating the use of ganciclovir for the treatment of symptomatic congenital infection by Whitley *et al* (1997) based on viral titres in urine. The results demonstrated a significant reduction in virus load in urine during therapy, which was associated with a more favourable clinical outcome in 16% of babies after six months follow up.

The use of quantitative PCR methodology for measuring virus load in the urine of congenitally infected infants provides a rapid and simple method for monitoring HCMV infection and antiviral therapy. The quantity of virus in the urine in infected infants gives an insight into the pathogenesis of HCMV replication in the presence of antiviral therapy. However, further studies are required with long term follow up and viral load measurement with respect to symptomatic and asymptomatic infants with or without antiviral therapy to establish the prognostic value of this technique.

Chapter 4.

A prospective analysis of HCMV viral load in renal transplant recipients: identification of risk factors for disease

4.1 Introduction

The pathogenesis of HCMV disease in renal transplant recipients still remains to be fully elucidated. The commonest disease manifestations of these patients are pneumonitis, hepatitis and more frequently persistent fever, and recipients are at the greatest risk of disease up to three months after transplant. Between 15% - 50% of patients excrete HCMV in their urine following renal transplantation, approximately 30% - 40% of whom develop HCMV disease (Balfour *et al*, 1989, Betts *et al*, 1977). With such a high incidence of HCMV disease, it is important to identify those patients at the greatest risk. Many studies have attempted to provide prognostic information by using various techniques including antigenaemia, culture, PCR and RT-PCR (Olive *et al*, 1989, Jiwa *et al*, 1989, Lee *et al*, 1992, Van Dorp *et al*, 1992, Bitsch *et al*, 1993, Kidd *et al*, 1993, Pillay *et al*, 1993b, Cunningham *et al*, 1995, Meyer-Konig *et al* 1995).

The more recent of these studies have utilised PCR in comparison with other methods in order to establish a methodology for identifying HCMV infection. The source of HCMV infection can be from either the kidney donor or the recipient. Primary infection (virus of donor origin infecting a non-immune individual) has been shown consistently to be a risk factor for HCMV disease. However, reactivation of latent recipient virus, has been associated with a significantly lower risk for disease (Betts *et al*, 1977, Grundy *et al*, 1988, Ranjan *et al*, 1991). Restriction-enzyme typing of HCMV strains excreted by pairs of recipients receiving kidneys from the same donor has shown that re-infection (donor virus infecting an immune individual) is associated with a risk of disease intermediate between primary infection and reactivation (Grundy *et al*, 1988). In addition several studies have shown that the detection of HCMV viraemia is a significant risk factor for HCMV disease (Betts *et al*, 1977, Cheeseman *et al*, 1979, Pillay *et al*, 1993b).

The sensitivity and specificity of qualitative PCR assays to provide prognostic information can render its use inappropriate as a marker for infection resulting in PCR positive results without further virological or clinical evidence of HCMV (Bitsch *et al*, 1993). Alternatively the level of sensitivity of the qualitative PCR assay can be set such that latent virus is not detected leading to improved prognostic value of qualitative PCR assays (Fox *et al*, 1992, Kidd *et al*, 1993). With respect to quantitative measures of HCMV in renal transplant recipients, recent, data generated using quantitative molecular techniques has highlighted the importance of measuring HCMV viral load for disease (Schafer *et al*, 1993, Fox *et al*, & 1995). Both Schafer (1993) and Kuhn (1994) *et al* demonstrated increased viral loads in peripheral blood leukocytes of patients with acute symptomatic

HCMV infection by nested PCR and temperature gradient gel electrophoresis. Fox *et al* (1995) showed the clinical significance of high viral loads in the urine of symptomatic renal transplant recipients by a quantitative competitive PCR assay.

From all the previous studies to identify risk factors for disease in renal transplant recipients, it is clear that no one parameter can be used to predict HCMV disease. The following chapter attempts to identify patients at the greatest risk of HCMV disease by examining the inter-relationships of various risk factors including viral load, donor/recipient serostatus and HCMV viraemia. Both qualitative and quantitative PCR methodologies were used to follow renal transplant patients prospectively for viral load modulations in urine, and where relevant their response to antiviral therapy. From this data set the probability of patients becoming symptomatic in relation to viral load and the presence of viraemia as a risk factor for disease is also presented.

4.2 Patients studied

4.2.1 Study population

Between July 1992 and December 1994, 196 renal transplant patients receiving their first renal transplant were followed prospectively for evidence of HCMV infection. Urine and blood samples were collected weekly from in-patients, and at each subsequent out-patient visit or where clinically indicated. Urine was analysed by PCR without processing, whereas HCMV in the blood was extracted using a commercial DNA extraction procedure (detailed in chapter 2 section 2.2.1 Qiagen, Germany) and subsequently detected by qualitative PCR for HCMV DNA (chapter 2 section 2.3). The term viraemia is used in this context to describe detection of HCMV DNA in blood by PCR. The total number of urine samples analysed from the transplant recipients was 2,968 (median number of samples per patient =7; range 1-62). The median age of the 35 patients subjected to detailed analysis and viral load quantification was 14 years (range 2-57). There was no significant difference in disease incidence in paediatric versus adult recipients ($p= 0.30$, Fisher's exact test) or between patients above or below the median age of 14 years ($p= 0.35$, Fisher's exact test).

4.2.2 Immunosuppressive therapy

The triple immunosuppressive regimen administered to adult and paediatric renal transplant recipients including the management of rejection episodes has been described previously (Irragori *et al*, 1993, Pillay *et al*, 1993). Briefly, paediatric transplant recipients received prednisolone ($60\text{mg}/\text{m}^2/\text{day}$ iv) pre-operatively reduced to $10\text{mg}/\text{m}^2/\text{day}$ by 4-6 weeks post transplantation. The same dose of prednisolone was administered but on alternate days by 8 weeks post-transplant. Patients also received azathioprine ($60\text{mg}/\text{m}^2$) 24 hours post-transplant and cyclosporin A ($50\text{mg}/\text{m}^2$ iv) pre-operatively then post-operatively maintaining plasma levels at 50-100ng/ml. Rejection episodes in the paediatric patients were treated with methylprednisolone ($600\text{ mg}/\text{m}^2/\text{day}$) for 3 days for the first 6 weeks post- transplant changing to high dose oral prednisolone ($3\text{ mg}/\text{kg}/\text{day}$) for 3 days thereafter. In the case of steroid resistant rejection, patients were treated with anti-thymocyte globulin or ATG ($2\text{ mg}/\text{kg}/\text{day}$) for 10 days. Adult transplant recipients received daily triple immunosuppressive therapy of prednisolone ($0.3\text{mg}/\text{kg}$), ATG ($1\text{mg}/\text{kg}$) and cyclosporin A ($10\text{-}15\text{ mg}/\text{kg}$). Initial rejection episodes were treated with methylprednisolone ($500\text{ mg}/\text{kg}/\text{day}$ iv) for 3 days subsequent episodes were treated with 1g methylprednisolone administered on three consecutive days. Further episodes of rejection were treated with ATG ($5\text{ mg}/\text{kg}$) for 10 - 15 days. Twenty-three adult recipients received chimeric anti-CD25 antibody in addition to their baseline immunosuppression

(Amlot *et al*, 1995).

4.2.3 Antiviral therapy

Six of the 35 patients analysed in detail were prescribed specific HCMV antiviral therapy comprising 3 patients treated with ganciclovir (5mg/kg/12 hrs bd iv) for 14-21 days adjusted according to renal function, 5 patients were administered aciclovir (5mg/kg td iv) for 7 days and one patient received both aciclovir and ganciclovir. Therapy was initiated at the onset of symptoms rather than pre-emptively in all cases. In addition, 3 patients received immunoglobulin for treatment of HCMV pneumonitis. None of the remaining 161 patients included in this study received any antiviral therapy.

4.3 Results

4.3.1 Qualitative PCR results

HCMV DNA was detected in the urine of 35 renal transplant recipients by PCR suggesting active viral replication. Twenty seven patients had three or more consecutive PCR positive samples 12 of whom developed disease (44.4%), none of the symptomatic patients had less than three PCR positive samples. Of the remaining 23 asymptomatic patients, 15 had at least three consecutive PCR positive samples, while the remaining eight patients had less than three urine samples which were positive for HCMV DNA in a non-consecutive sequence two of whom developed disease ($p=0.021$, Fisher's exact test). The remaining 161 patients were PCR-negative in urine and asymptomatic. Viraemia as detected by HCMV PCR in blood was positive in fourteen patients (40%) 10 of whom developed disease all of which were also PCR positive in urine. In contrast, only 2 (5.7%) of the none viraemic patients developed HCMV disease ($p=0.00052$, Fisher's exact test). None of the remaining 146 patients were HCMV viraemic.

4.3.2 Quantitative PCR results

All thirty five renal transplant recipients that were PCR positive in urine during post-transplant surveillance were subjected to detailed study of virus load using a quantitative competitive PCR assay (QC-PCR) described in chapter 2 section 2.4. Twelve of these patients exhibited HCMV disease. The period between transplant and onset of disease in these patients was 11, 12, 21, 24, 32, 33, 36, 36, 75, 99, 120 and 370 days post-transplant. The median number of urine samples analysed by quantitative PCR from the 35 patients was 12 (range 4 - 39) which was comparable between asymptomatic ($n=23$; median number of samples per patient =10; range 4 - 36) and symptomatic patients ($n=12$; median number of samples per patient =14; range 4 - 39). The maximum viral load present in the urine during the post-transplant surveillance period ranged between $10^{3.1}$ and $10^{7.1}$ genomes/ml of urine (median = $10^{5.2}$ genomes/ml). Details of the maximum viral load for each patient together with the donor/recipient serostatus and whether viraemia was present are detailed in table 4.1. Representative longitudinal viral load profiles from 6 symptomatic and 6 asymptomatic patients with viruria are shown in figure 4.1 and figure 4.2 respectively. Viral load was plotted as a function of time together with the clinical course of the patients, antiviral therapy where applicable and total white blood cell count.

Table 4.1 Maximum viral load in urine, disease status and detection of HCMV viraemia in the 35 renal transplant recipients patients who were HCMV PCR positive in urine following renal transplantation.

HCMV Disease	Maximum viral load log ₁₀ genomes/ml urine for each patient					
	Viraemic			Non-viraemic		
	D+/R-	D+/R+	D-R+	D+/R-	D+/R+	D-R+
Yes	7.1	6.85	-	5.9	-	-
	6.9	6.6				
	6.85	6.33				
	6.55	6.3				
	6.2					
	5.85					
	5.8					
No	5.25	3.55	3.7	6.55	5.55	4.85
	4.0			5.75	4.14	4.55
				5.5	3.95	4.25
				5.3	3.8	3.7
				5.3	3.75	3.6
					3.7	3.1
					3.1	3.1

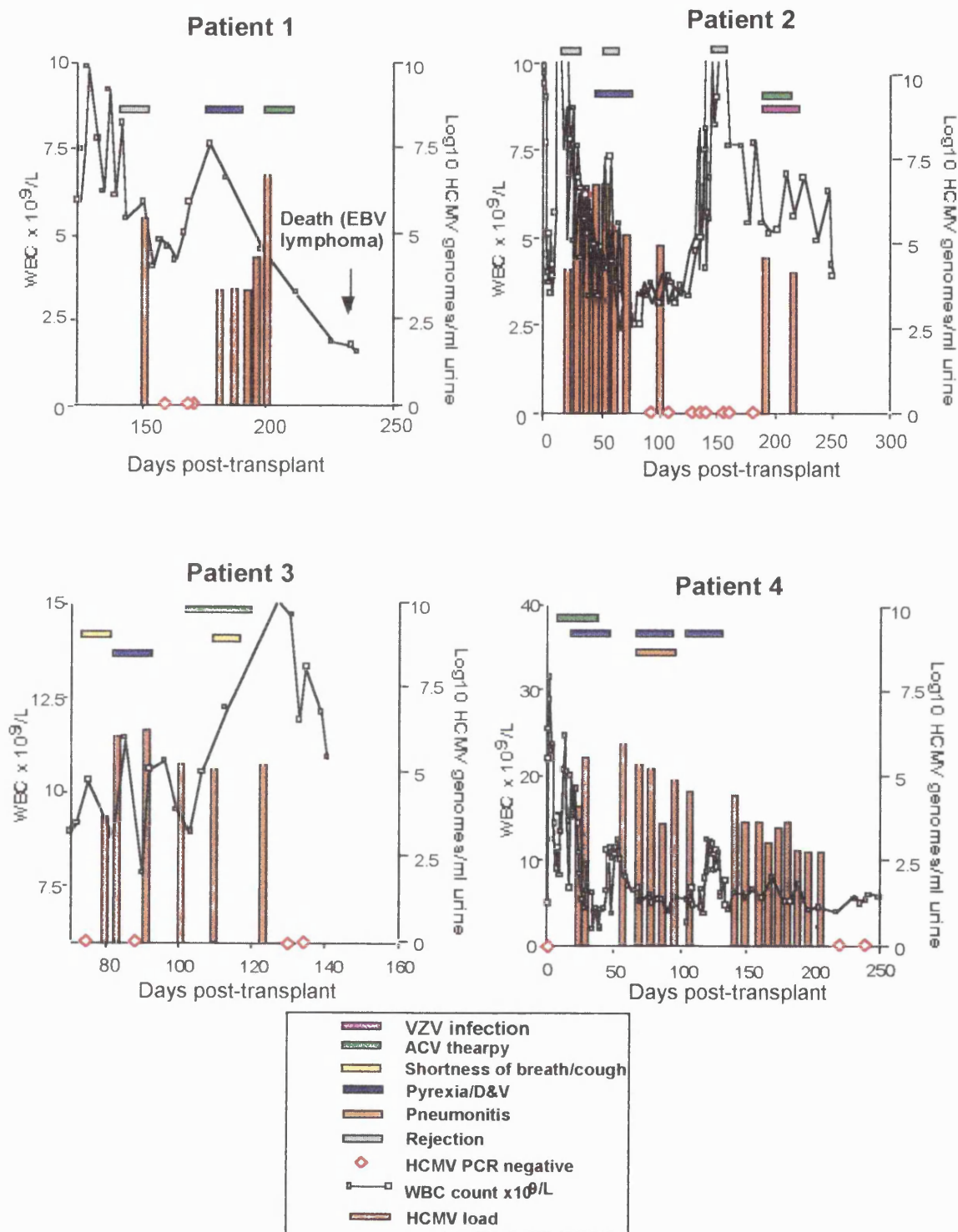


Figure 4.1 Representative longitudinal HCMV viral load profiles in urine from 6 symptomatic renal transplant recipients. Total white blood cell count and the clinical course of the patient are also indicated, (where the normal range for total white blood cells is $4.0-11.0 \times 10^9/L$) together with the administration of antiviral therapy where applicable.

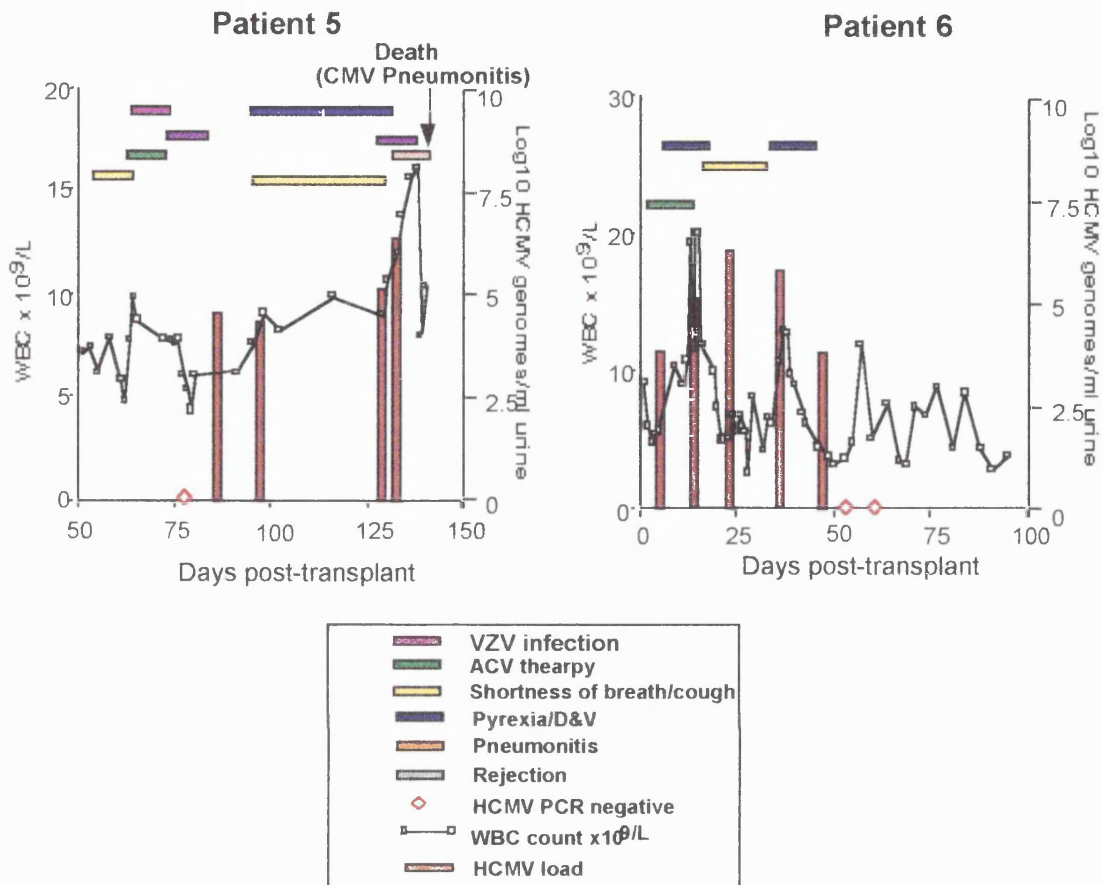


Figure 4.1 Continued

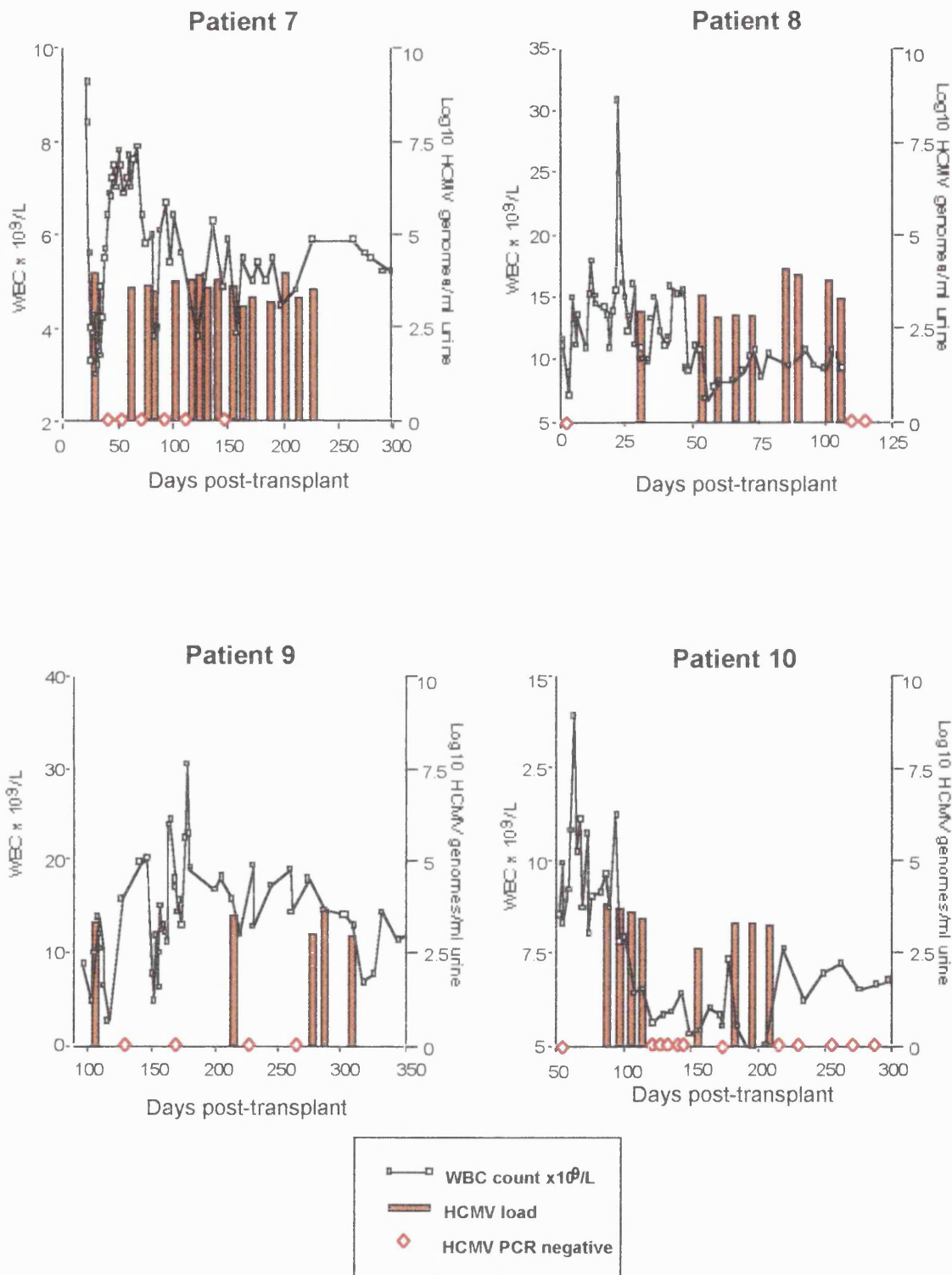


Figure 4.2 Representative longitudinal HCMV viral load profiles in urine from 6 asymptomatic renal transplant recipients, with total white blood cell count.

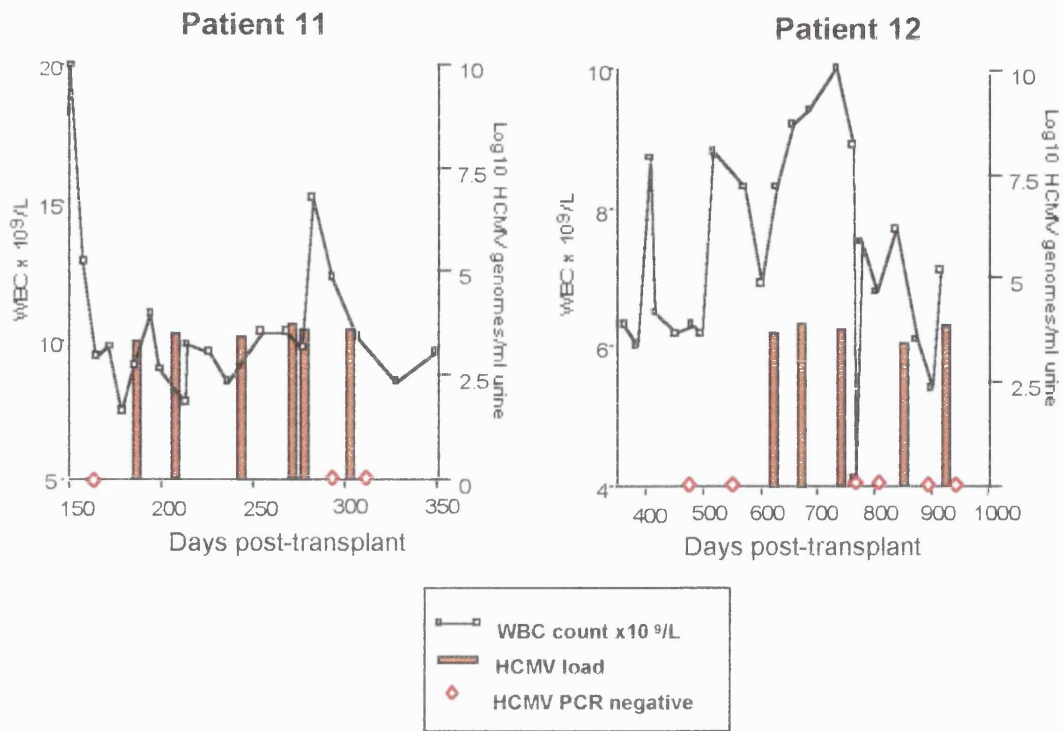


Figure 4.2 Continued

4.3.3 Relationship between HCMV viral load and disease

Statistical analysis was performed on the quantitative viral load measurements from the 35 renal transplant recipients in order to establish the relationship between viral load and HCMV disease. The maximum viral loads from patients experiencing disease and those patients who remained asymptomatic were compared and plotted as a scatter diagram shown in figure 4.3. The median peak viral load in the symptomatic patients was $10^{6.44}$ genomes/ml urine (n= 12; range $10^{5.8} - 10^{7.1}$) which was significantly higher than the median viral load in asymptomatic patients (n=23: median = $10^{4.0}$ genomes/ml urine, range $10^{3.1} - 10^{6.55}$; $p < 0.01$, Mann-Whitney U test). The difference between medians corresponded to 2.44 \log_{10} genomes/ml urine or was 275-fold higher in symptomatic patients compared to asymptomatic patients.

4.3.4 Relationship between HCMV viral load and viraemia

A similar analysis was performed to investigate the relationship between maximum viral load, disease and the detection of HCMV viraemia. The results are presented as a scatter diagram shown in figure 4.4. The median viral load of the viraemic patients was $10^{6.3}$ genomes/ml urine (n=15; range $10^{3.55} - 10^{7.10}$) which was significantly higher than the non-viraemic patients with a median of $10^{4.20}$ genomes/ml (n=20; range $10^{3.1} - 10^{6.55}$). Patients with viraemia had a 125-fold higher median viral load compared to the non-viraemic patients ($\log_{10} 2.10$ genomes/ml urine), which was statistically significant according to the Mann-Whitney U test ($p < 0.01$). Only one of the non-viraemic patients experienced HCMV disease with a viral load above the median of the asymptomatic group. Whereas 11 of the viraemic patients had HCMV disease (73.3%), six of these patients had maximum viral loads above the median viral load for the symptomatic group. The remaining five patients had viral loads below the median of the symptomatic group implying that a lower was required to cause disease in these viraemic patients.

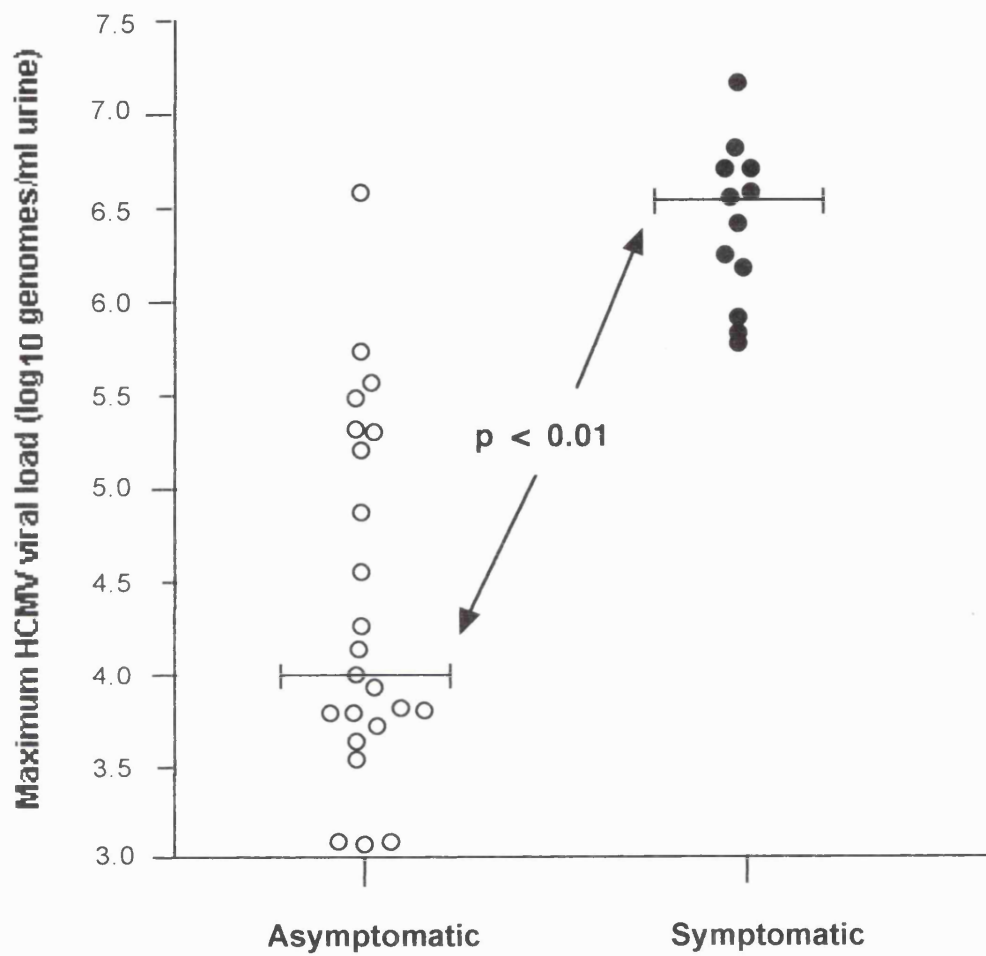


Figure 4.3 Relationship between maximum HCMV viral load in the urine of 35 renal transplant recipients and HCMV disease. The closed circles represent patients with disease, the open circles depict asymptomatic patients.

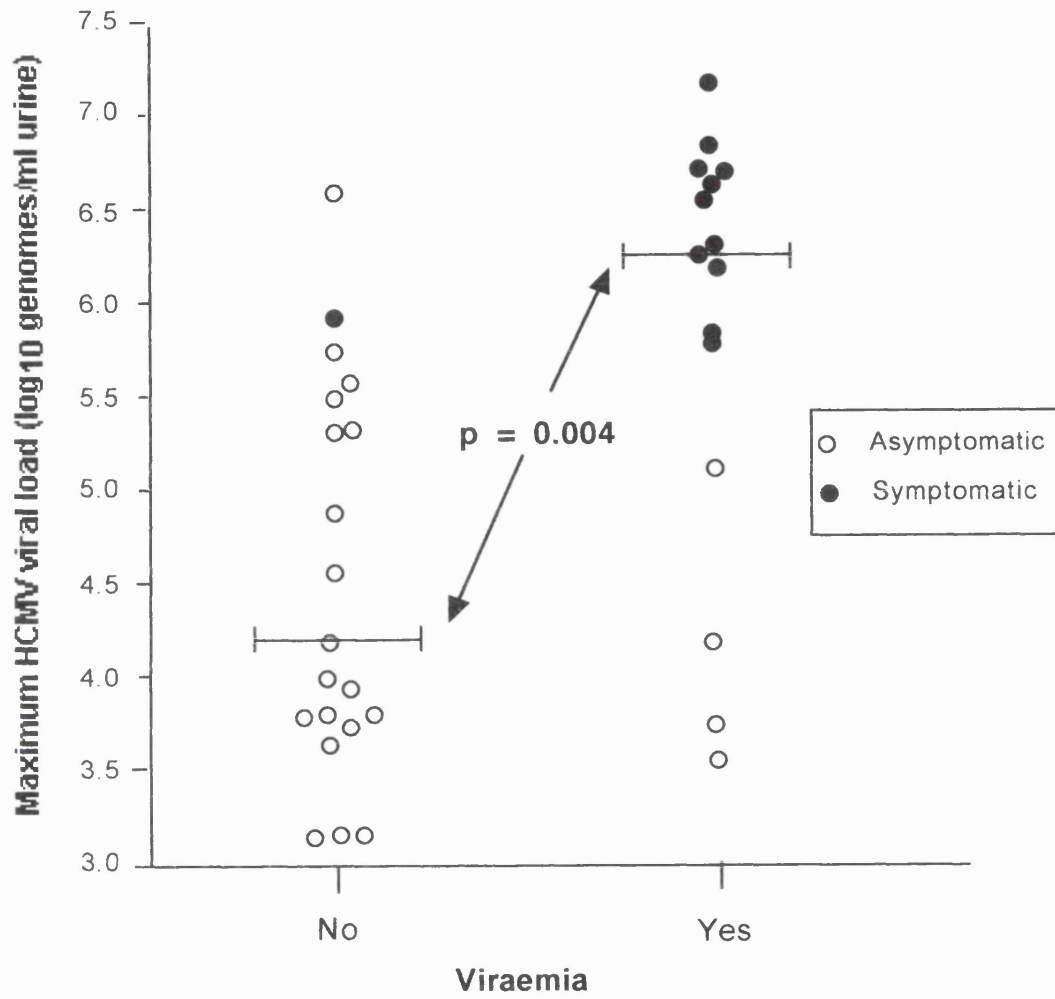


Figure 4.4 Relationship between maximum HCMV viral load in the urine of 35 renal transplant patients, HCMV disease and the detection of HCMV viraemia

4.3.5 Relationship between HCMV viral load and donor/recipient serostatus

The relationships between maximum HCMV load in the urine post-transplantation with HCMV disease and donor/ recipient serostatus is shown in figure 4.5. As with the previous analyses the data was plotted as a scatter diagram where patients with and without HCMV disease were segregated according to their HCMV serostatus. The primary infection (D+R-) group had the highest median viral load of $10^{5.85}$ genomes/ml urine (n=15; range $10^{4.05} - 10^{7.01}$) and the most patients with disease (53.3%) when compared with the other two groups. The reactivation group (D-R+) had the lowest median viral load of $10^{3.73}$ genomes/ml urine (n=8; range $10^{3.1} - 10^{6.1}$) and no patients experienced HCMV disease in this group. Patients in the D+R+ group had a median viral load that was intermediate between the two aforementioned groups at $10^{4.03}$ genomes/ml urine (n=12; range $10^{3.1} - 10^{6.85}$) with four of these patients experiencing HCMV disease (33.3%). When analysed statistically using the Mann-Whitney U test for the differences between medians of the three groups, viral load in the primary infection group was significantly higher than the viral load in patients reactivating their own latent virus (D+R- group versus D-R+, $p < 0.01$) corresponding to a 132-fold difference, and in patients at risk of either reactivation or re-infection (D+R- versus D+R+ group, $p < 0.05$) corresponding to a 66-fold increase. There was no significant difference between patients reactivating their own virus (D-R+) and patients at risk of either reactivation or reinfection (D+R+).

Interestingly, in those patients at risk of either reactivation or re-infection (D+R+) two viral load populations were apparent; the first population none of whom had disease had a viral load comparable to the median levels detected in the D-R+ group (n=7; median $10^{3.8}$ genomes/ml, range $10^{3.1} - 10^{4.15}$). The other population exhibited a significantly higher HCMV load (n=5, median $10^{6.33}$ genomes/ml, range $10^{5.6} - 10^{6.85}$; $p < 0.01$ Mann-Whitney U test) which was comparable to the median viral load detected in the D+R- group suggesting a greater risk of disease associated with re-infection of donor strains of the virus.

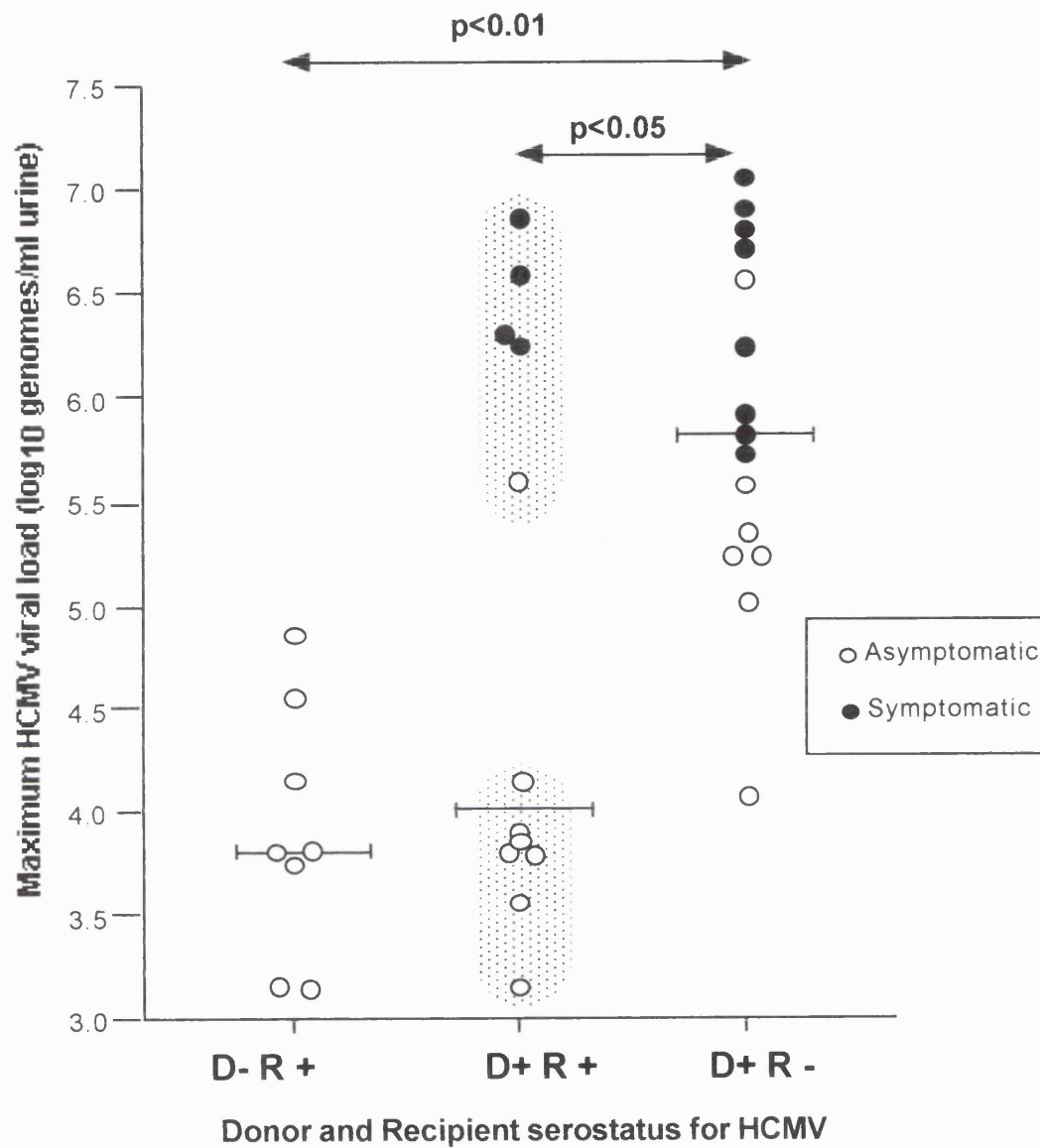


Figure 4.5 Relationship between HCMV viral load, HCMV disease and HCMV serostatus in 35 renal transplant recipients, where the shaded area represents the two populations within the D+R+ group at risk of either reactivation of endogenous HCMV or reinfection of exogenous HCMV strains.

4.4 Quantification of risk factors for HCMV disease

4.4.1 Univariate statistical analysis

Increased viral load in urine, viraemia and receipt of an organ from a seropositive donor were all significantly associated with an increased risk of disease ($p=0.0001$, $p=0.0003$ and $p=0.03$ respectively Fisher's exact test), whereas being HCMV seropositive was associated with a lower risk of HCMV disease ($p=0.04$, Chi-squared test). In order to quantify the identified individual risk factors of viral load, viraemia and donor/recipient serostatus, univariate analysis was performed. This type of statistical analysis takes each risk factor as an independent continuous variable where the odds ratios refer to the likelihood of disease in those patients with the factor of interest versus those patients within the same data set without that risk factor .

The results of the univariate logistic regression model are shown in table 4.2. There was a significant association between increased viral load and disease ($p=0.02$) with an odds ratio of 2.79 per $0.25\log_{10}$ increase in viral load. In addition, viraemia was also a highly significant risk factor for HCMV disease ($p=0.0009$) with an odds ratio of 23.75. The protective effect of being recipient seropositive for HCMV also reached statistical significance ($p=0.05$) with an odds ratio of 0.22. The risk associated with receiving an organ from a seropositive donor could not be quantified as a risk factor in this type of analysis since there were no patients in the D-R+ group with HCMV disease, hence the odds ratio was infinity and so was not included in further analysis.

4.4.2 Multiple logistic regression analysis

To test the hypothesis that the aforementioned risk factors were dependent variables which interact with each other, multiple logistic regression analysis was performed for viral load, viraemia and recipient seropositivity. This type of analysis allows one of the established risk factors to remain constant while the others are simultaneously controlled for in a bivariate model. In the multivariate analysis, adjusting for viraemia affected the odds ratio associated with viral load only marginally (OR 2.77; $p=0.04$) thus maintaining the significant effect of viral load (detailed in table 4.2). When viral load was controlled with respect to viraemia, the significance of viraemia as a risk factor was markedly reduced despite increasing the odds ratio from 23.75 to 34.54 ($p=0.07$ from $p=0.0009$ in the univariate model). The significant protective effect of recipient seropositivity against HCMV disease was negated once either viral load or viraemia had been controlled for ($p=0.98$).

Table 4.2 Univariate and multivariate analysis relating the odds ratio for HCMV disease with viral load, viraemia and recipient serostatus for HCMV.

Parameter	Univariate analysis			Multivariate analysis		
	Odds ratio	95% C.I.	p-value	Odds ratio	95% C.I.	p-value
Viral load (per 0.25 log ₁₀ increase)	2.79	1.22 - 6.39	0.02	2.77	1.07 - 7.18	0.04
Viraemia	23.75	3.69 - 153	0.0009	34.54	0.75 - 1599	0.07
Recipient seropositivity	0.22	0.05 - 0.95	0.05	0.92	0.002 - 446	0.98

4.4.3 Computed Probability of HCMV disease

Based on the data generated from the univariate and multivariate analysis, an equation was constructed to calculate the probability of suffering from HCMV disease at specific viral loads. The univariate analysis was used to construct the disease-probability curve shown in figure 4.6. which illustrates the expected probability of suffering HCMV disease at any given viral load for all 35 patients subjected to HCMV viral load quantification. The graph shows a sharp transition in risk of disease for a relatively modest increase in viral load from between $10^{5.5}$ and $10^{6.0}$ genomes/ml urine i.e $0.5 \log_{10}$ increase. Thus, at lower viral loads (below $10^{5.0}$ genomes/ml urine) the probability of disease was less than 10%, which increased to a disease probability of 70% at 10^6 genomes/ml urine i.e over a $1.0 \log_{10}$ increase. A similar graph was constructed based on the multivariate analysis and is shown in figure 4.7. In this analysis the two viral load probability of disease curves represent patients with HCMV viraemia and non-viraemic patients. The major effect of viraemia was to shift the disease probability curve such that disease in these patients occurred at lower viral loads than in patients who were non-viraemic. When comparing viral loads in these two groups for a 50% probability of disease, the viral loads were separated by $0.8 \log_{10}$ genomes/ml urine i.e for viraemic patients the 50% probability of disease was reached at $10^{5.4}$ genomes/ml compared to a viral load of $10^{6.2}$ genomes/ml in the non-viraemic patients.

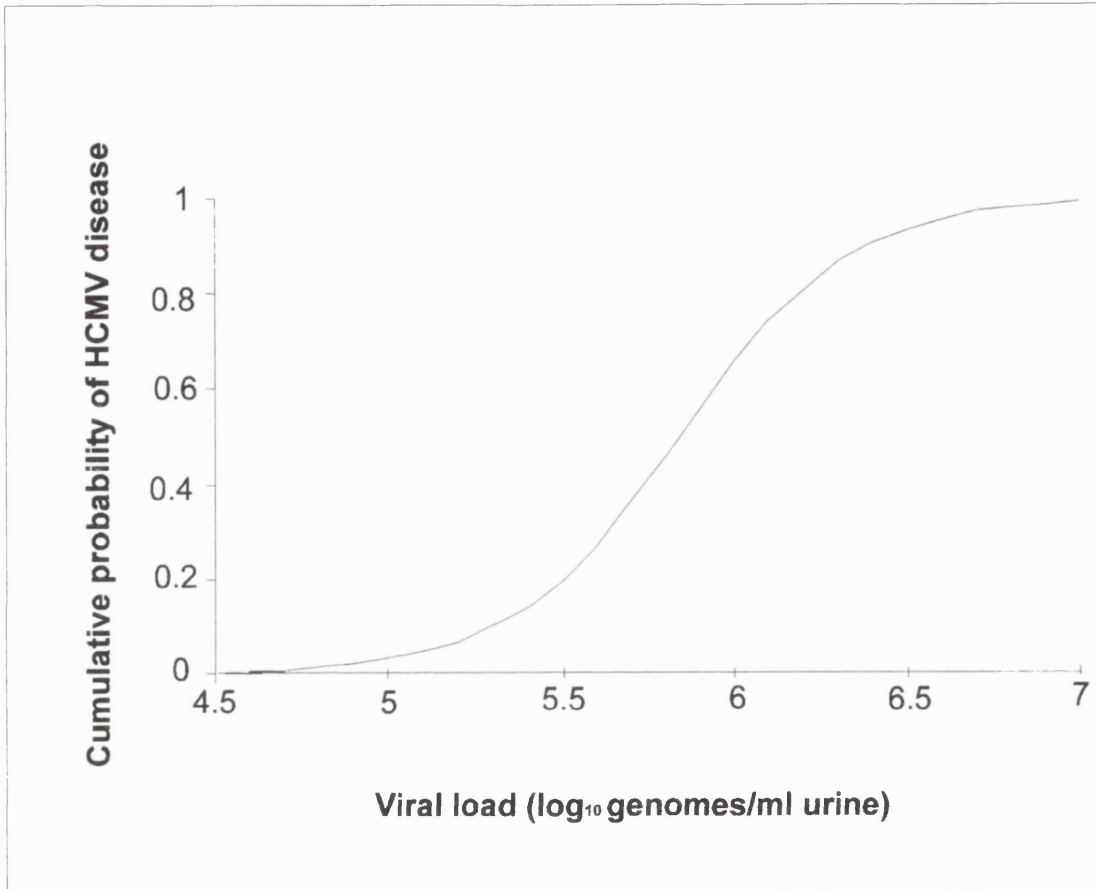


Figure 4.6 Probability of HCMV disease with increasing viral load estimated from the univariate logistic regression analysis.

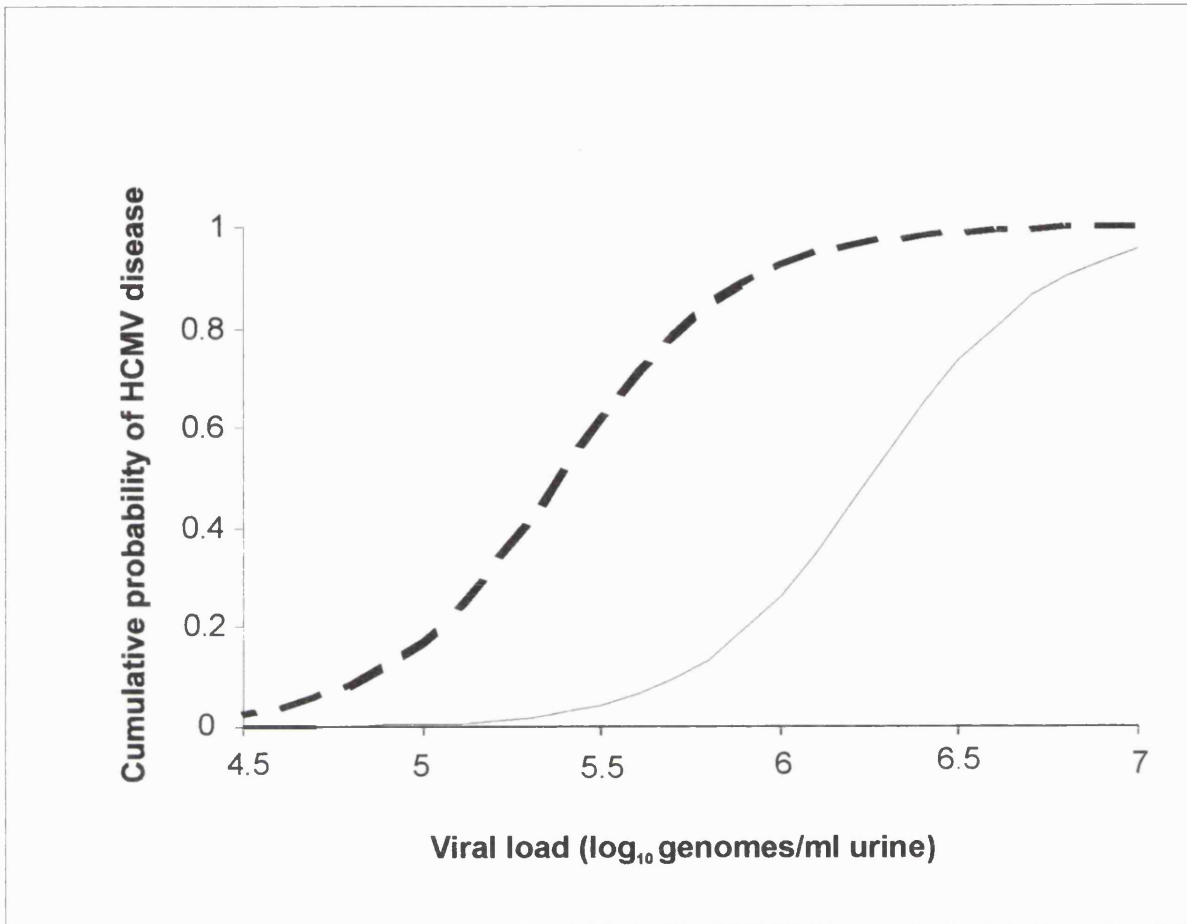


Figure 4.7 Probability of HCMV disease with increasing viral load for viraemic (dotted line) and non-viraemic (solid line) patients estimated from the multivariate logistic regression analysis.

4.5 Discussion

The results of this study clearly demonstrate that the quantity of viraemia, rather than its mere presence post-renal transplant, is a significant risk factor for HCMV disease. Thus, the median peak viral load in the urine post-transplant was elevated in patients experiencing symptomatic infection, those with primary HCMV infection, and in patients with viraemia. Elevated viral load in urine, viraemia and receipt of an organ from a seropositive donor were all significantly associated with disease in the 35 patients subjected to detailed quantitative HCMV measurements. Univariate analysis established elevated viral load and viraemia as risk factors for disease, whereas recipient seropositivity had a protective effect against HCMV disease. Hence, patients in this study are representative of those previously reported in the literature as they have similar risk factors for disease (Betts *et al*, 1977, Goodrich *et al*, 1991, Ranjan *et al*, 1991, Irragorri *et al*, 1993, Schafer *et al*, 1993, Khun *et al*, 1994, Fox *et al*, 1995,).

The dominant influence of viral load determined in this longitudinal study can be used to provide insight into the HCMV pathogenesis. For example, the observation that the temporal patterns of disease and elevated viral loads were coincident provides an explanation for the results of cross-sectional studies showing that HCMV load is higher in patients with disease (Kuhn *et al*, 1994, Rasmussen *et al*, 1995, Saltzman *et al*, 1992, Schafer *et al*, 1993). The observation that two viral load populations exist within the D+R+ group helps explain the intermediate value for the risk of HCMV disease previously observed in this donor/recipient patient subgroup. Thus, individuals with low virus load (comparable to D-R+ individuals) were likely to be experiencing reactivation of their own HCMV and so have a reduced probability of disease. In contrast, the other group comprised those individuals with an increased level of HCMV load, presumably reflecting reinfection with donor HCMV strains which is associated with a higher probability of disease (Grundy *et al*, 1988). Although this suggestion requires further support from restriction fragment length polymorphism studies, it potentially allows the identification of patients experiencing reinfection without requiring the donor strains of virus. Since donor kidneys are in short supply, and over 50% of donors are HCMV seropositive, it would be impracticable to reject infected donors, as is done for hepatitis B, human immunodeficiency virus, and hepatitis C. Hence, transplantation of HCMV-positive organs into HCMV seronegative recipients continues to occur, and clearly these patients are at highest risk of both HCMV infection and disease.

To analyse the relative contributions of viraemia, elevated viral load and recipient

seropositivity to HCMV disease, multivariate logistic regression analysis was performed. In this analysis viral load remained a significant risk factor for HCMV disease i.e. it was only marginally influenced by viraemia, whereas the significance of viraemia as a risk factor was substantially reduced after controlling for viral load in urine. The protective effect of recipient seropositivity against disease observed in univariate logistic regression analysis was negated once either viral load or viraemia were controlled for. In contrast, recipient seropositivity had no marked effect on odds ratios for either viral load or viraemia in the multivariate analysis. Although the data show the dominant role of elevated viral load as an independent risk factor to viraemia they do not exclude the possibility of an associated effect of viraemia.

The relationship between the probability of having HCMV disease and increasing viral load illustrates that at critical viral load levels, small increases in viral load resulted in a substantial increase in the probability of disease. When considering the effect of viraemia, the effect was to shift the disease probability such that disease occurred at lower viral loads when compared to the non-viraemic patients. These findings have implications for understanding the pathogenesis of HCMV disease, the early identification of patients at risk of HCMV disease and in the design of strategies aimed at reducing the incidence and severity of HCMV disease and will be discussed fully in chapter 8.

In order to reduce HCMV disease after renal transplant various strategies involving the use of antiviral drugs and/or vaccines have been considered including prophylaxis with α -interferon (Cheeseman *et al*, 1979, Hirsch *et al*, 1983, Lui *et al*, 1992), aciclovir (Balfour *et al*, 1989), immunoglobulin (Metselaar *et al*, 1989) or vaccination with a live, attenuated strain of HCMV (Plotkin *et al*, 1991). Two more recent trials with prophylactic ganciclovir significantly reduced the incidence of HCMV disease (Conti *et al*, 1994, Hibberd *et al*, 1995) thus, limiting the exposure of the drug to those patients at greatest risk. In other transplant patient groups ganciclovir has been successfully as prophylaxis or pre-emptive therapy (Schmidt *et al*, 1991, Goodrich *et al*, 1991).

It can be suggested that the initial identification of risk factors for disease such as susceptibility to primary infection and detection of viraemia, together with constant monitoring of patients for viral load could be used to direct treatment regimens to control HCMV disease in renal transplant patients. Furthermore, the use of pre-determined viral load threshold levels as a proximal marker of disease in association with other risk factors for disease, such as viraemia, could potentially provide comparative data on the

relative efficacy of existing and new antiviral interventions. For example, immunisation schedules with prototype vaccines or prophylactic schedules of safe antiviral drugs could be optimised to keep virus loads below a defined threshold, without necessarily aiming to eradicate HCMV infection completely. Clearly, the objective of reducing the level of virus replication should be easier to attain than complete elimination of all replication. Drugs with potent anti-HCMV activity could then be reserved for those with high viral loads or those predicted to attain high loads, thus minimising the number of patients exposed to their toxic effects. On the basis of the results presented here, a relatively modest reduction in viral load within the range of $10^{5.5}$ to $10^{6.0}$ could have a significant effect on disease. In addition, threshold viral load values could be used as laboratory markers to identify patients with a high risk of future disease, and the ability to use urine for this purpose has obvious practical advantages for routine monitoring. By extension, the same principles could be applied to other patients at risk of HCMV disease and similar analysis for liver transplant recipients is presented in chapter 5.

Chapter 5.

Inter-relationship between HCMV viral load in blood, donor/recipient serostatus and administration of methylprednisolone as risk factors for HCMV disease following liver transplantation.

5.1 Introduction

Human cytomegalovirus is a recognised cause of both morbidity and mortality following solid organ transplantation. HCMV is the most common cause of infection following liver transplantation with an incidence of 20-60% and in the absence of therapeutic intervention approximately 50% of infected patients develop HCMV disease (Paya *et al*, 1989, Stratta *et al*, 1989). The onset of symptoms associated with HCMV infection appear typically within the first three months post-transplant as reported for other solid organ transplant recipients, but occurs sooner in patients with primary infection (Falagas *et al*, 1996). Clinical manifestations range from persistent fever and a mononucleosis-like syndrome to more severe invasive organ disease such as HCMV hepatitis, gastrointestinal disease and pneumonitis. (Rubin *et al*, 1993).

The most clearly defined risk factors for HCMV disease are transplantation of an organ from a HCMV-seropositive donor into a HCMV-seronegative recipient, and the appearance of viraemia (Paya *et al*, 1989, Sutherland *et al*, 1992, Falagas *et al*, 1996, Badley *et al*, 1996). Several studies have also identified receipt of large doses of immunosuppressive therapy for rejection as a significant risk factor for HCMV disease and secondary infections (Stratta *et al*, 1992, Portela *et al*, 1995, Hadley *et al*, 1995, Lao *et al*, 1997). There are two possible explanations for the association of HCMV infection with rejection; one that the virus predisposes an individual to rejection, or that the use of increased doses of immunosuppression to treat graft rejection may render the individual susceptible to HCMV infection.

Rapid and reliable techniques to monitor patients for HCMV infection are required in order to direct antiviral therapy and allow the correct management of immunosuppressive therapy. The gold standard methods of conventional cell culture and histology although reliable, are time consuming and can be inhibited by the use of antiviral prophylaxis (Colina *et al*, 1995, Manez *et al*, 1994). These methods were improved upon by the shell vial assay and rapid detection of DNAemia using PCR techniques. PCR has proved in several studies to be a highly sensitive and specific detection for HCMV infection (Kidd *et al*, 1993, Wolf *et al*, 1993, Schmidt *et al*, 1993 & 1995, Patel *et al*, 1994, Nydberg *et al*, 1994, Manez *et al*, 1996, Lao *et al*, 1997). Indeed, many studies have confirmed that detection of HCMV DNA by PCR of plasma, serum and whole blood can provide prognostic information in liver transplant recipients (Wiens *et al*, 1993, Schmidt *et al*, 1993, Nydberg *et al*, 1994, Patel *et al*, 1994 & 1995, Schmidt *et al*, 1995, Manez *et al*,

1996, Lao *et al*, 1997, Mutimer *et al*, 1997).

As stated in chapter 4 the relationship between increased viral load and the development of HCMV disease in the renal transplant setting has been established (Kuhn *et al*, 1994, Fox *et al*, 1995). In the case of liver transplant recipients there have been limited longitudinal studies to examine the relationship between virus load and disease. One recent study, however, using a semi-quantitative PCR assay to follow 40 liver transplant patients, showed a significant correlation between high levels of HCMV DNA in the blood and development of disease (Drouet *et al*, 1995). Mutimer *et al* (1997) used a quantitative competitive PCR assay to examine the viral titre present in sera from 15 liver transplant recipients with symptomatic HCMV infection. This study identified increased viral titre in sera as a risk factor significantly associated with symptoms in all cases. Viral titre fell upon resolution of symptoms with reduced immunosuppression and administration of antiviral therapy. As yet however, no detailed longitudinal studies have been performed in liver transplant recipients using fully quantitative PCR methods to determine the relative contributions of viral load in the context of other risk factors for disease.

With the development of effective antiviral agents against HCMV, the emphasis has focused more recently on earlier intervention strategies to reduce incidence of disease (Stratta *et al*, 1992, Paya *et al*, 1993, Van den Berg *et al*, 1993, Singh *et al*, 1994, Snyderman *et al*, 1994, Winston *et al*, 1995b). The use of prophylactic ganciclovir versus high-dose aciclovir in controlled trials has shown ganciclovir to be more effective at preventing HCMV disease (Paya *et al*, 1993, Winston *et al*, 1995b). Indeed, one randomised trial has shown short-course pre-emptive ganciclovir therapy to be more effective than high-dose aciclovir (Singh *et al*, 1994).

This chapter details longitudinal viral load measurements in the blood of 47 liver transplant patients and relates viral load to the clinical course of the patients. The effects of antiviral therapy and inter-relationships with donor/recipient seropositivity and receipt of augmented immunosuppression for rejection episodes were also assessed in the context of HCMV disease.

5.2 Patients Studied

5.2.1 Study population

Between November 1992 and April 1996, 162 patients with a total of 1,433 surveillance blood samples were followed prospectively for HCMV infection using qualitative PCR. The median age of the patients was 50 years (range 9-70 years) and was not significantly different between asymptomatic and symptomatic patients. Samples of urine and blood were taken weekly until discharge, at subsequent regular out-patient visits and where clinically indicated, and were tested by conventional cell culture (CCC) and PCR for HCMV DNA. Liver biopsies were taken as clinically indicated and processed for histology and cell culture by detection of early antigen fluorescent foci (DEAFF). Pre-transplant sera from donors and recipients were tested for the presence of IgG antibodies to HCMV by enzyme immunoassay (Biokit SA, Spain). All recipients were also tested for HSV IgG antibodies by enzyme immunoassay (Biokit SA, Spain) according to the manufacturer's instructions.

5.2.2 Immunosuppressive therapy

Triple immunosuppressive therapy was initiated immediately after transplant, consisting of azathioprine (1.5 mg/kg daily), methylprednisolone (0.8 mg/kg daily) and cyclosporin A (4 mg/kg/24hr iv) from days 1 - 3 post-transplant, adjusted according to plasma levels and renal function of the patient. Moderate to severe rejection, diagnosed histologically, was treated with methylprednisolone (1g daily iv for 3 days), which was repeated upon subsequent rejection episodes. Steroid-resistant rejection was treated with either OKT3 for 5 days or ATG for 10 days and cyclosporin was replaced with FK506 (0.1 mg/kg daily) in the case of chronic rejection.

5.2.3 Antiviral and antimicrobial chemotherapy

Ampicillin (1g qds iv), netilmicin (3.5 mg/kg iv bd) and metronidazole (500 mg tds iv) were given for bacterial prophylaxis and amphotericin (5 ml qds) for fungal prophylaxis. No antiviral prophylaxis or pre-emptive therapy was administered for HCMV infection. However, patients who were HSV-antibody-positive received aciclovir prophylaxis (5 mg/kg tds iv initially followed by 200 mg qid orally) for at least one month after transplantation. In patients with diagnosed HCMV disease, immunosuppressive therapy was reduced and ganciclovir therapy was administered (5 mg/kg bd iv) adjusted according to renal function. In the case of HCMV pneumonitis, human immunoglobulin was given intravenously, in addition to ganciclovir. Patients who did not respond to

ganciclovir were treated with foscarnet (60 mg/kg q8 or 90/mg/kg q12). All patients received either ganciclovir or foscarnet following the diagnosis of HCMV disease according to the criteria outlined in chapter 2 section 2.9.

5.3 Results

5.3.1 Qualitative PCR results

Prospective follow-up of 162 liver transplant patients by qualitative PCR for HCMV from 1,433 blood samples identified 51 patients with HCMV viraemia i.e the detection of HCMV DNA in blood. The median number of PCR positive samples from the 51 patients was 9 (range, 1- 24). Seventeen patients had three or more PCR positive blood samples, 11 patients were PCR positive in non-consecutive samples while the remaining 23 patients had a single PCR positive sample. Twenty of the 51 viraemic patients (40.4%) experienced HCMV disease, according to the criteria outlined in chapter 2 section 2.9, compared with none of the remaining 111 PCR negative patients (Fisher's exact test; $p < 0.0001$). The number of PCR positive blood samples in the 20 patients with HCMV disease (median= 3.5, range 1 - 11) was significantly higher compared to the remaining 27 asymptomatic recipients (median = 1, range 1 - 4, $p < 0.0001$, Mann Whitney U test).

5.3.2 Quantitative PCR results

Forty seven of the fifty one patients with HCMV viraemia were followed in detail by quantitative competitive PCR for HCMV viral load in blood. Four asymptomatic patients with single PCR positive blood samples that contained insufficient DNA in the sample for quantification were excluded. The number of days between the date of transplant and onset of disease in the symptomatic patients was 20, 22, 31, 32, 34, 35 (n=2), 40 (n=2), 43 (n=2), 50, 59, 60 (n=2), 65, 90 (n=2) and 126 days post-transplant. Maximum viral loads from all 47 patients are detailed in table 5.1 together with the corresponding donor/recipient serostatus and whether patients experienced HCMV disease. The maximum viral load detected during the post-transplant surveillance period ranged between $10^{3.59}$ to $10^{7.57}$ genomes/ml blood (median= $10^{4.91}$ genomes/ml blood). Representative longitudinal HCMV viral load profiles with corresponding alanine aminotransferase (ALT) levels of 4 asymptomatic liver transplant recipients are shown in figure 5.1. Representative viral load profiles from 6 of the 20 patients with HCMV disease together the administration of antiviral therapy and the clinical course of the patients are shown in figure 5.2.

Table 5.1 Maximum HCMV viral load in blood, donor/recipient serostatus and presence of HCMV disease in 47 liver transplant recipients with HCMV viraemia.

HCMV Serostatus	Maximum HCMV viral load log ₁₀ genomes/ml blood	
	HCMV Disease	
	Yes	No
D+/R-	7.57, 6.54, 6.48, 6.46, 6.31, 6.28, 5.65, 5.63, 5.43, 5.34, 5.25, 5.01	4.8, 4.74, 4.23, 4.12, 3.59
D+/R+	7.53, 6.36, 6.33, 5.57, 5.11, 4.95, 4.94	5.3, 5.18, 5.17, 4.92, 4.87, 4.6, 4.56, 4.15, 3.91, 3.9, 3.88, 3.64
D-/R+	3.66	4.92, 4.62, 4.56, 4.46, 4.35, 4.0, 4.0, 4.0, 3.84, 3.74

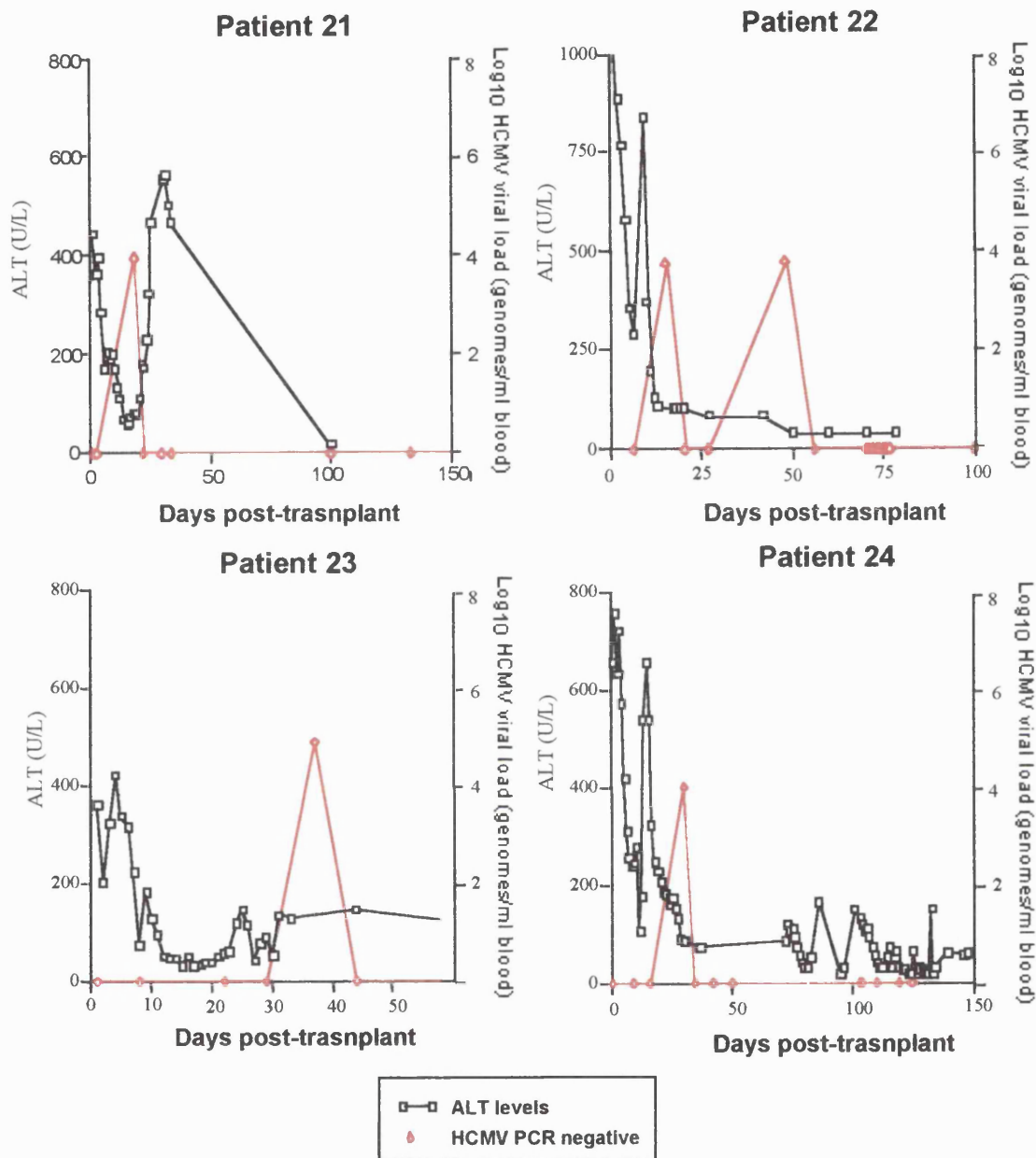


Figure 5.1 Representative HCMV viral load profiles from 4 asymptomatic liver transplant recipients. For each patient, the donor/recipient serostatus is shown together with the corresponding ALT levels where the normal range is 5.0 - 40.0 U/L (units/litre).

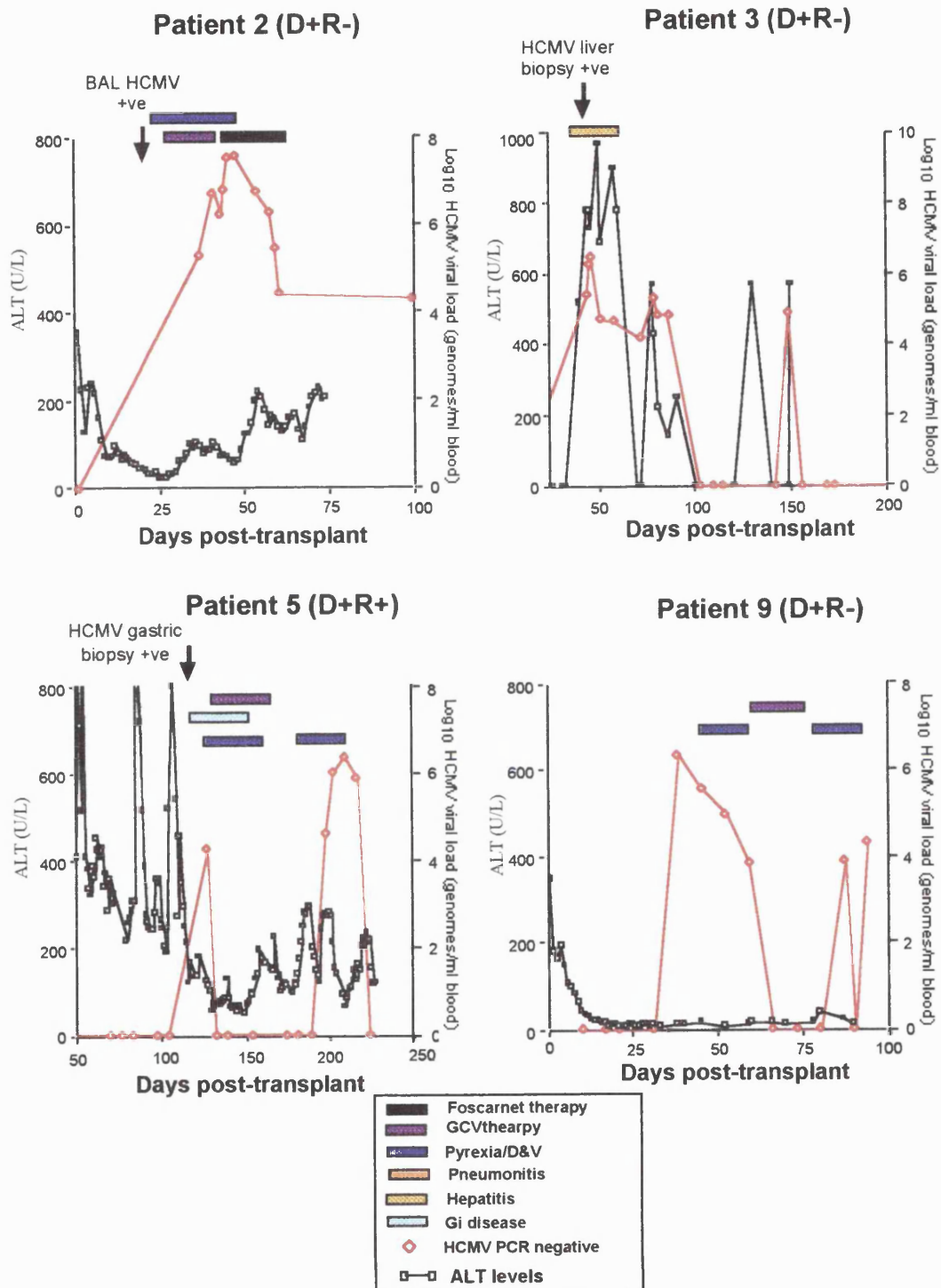


Figure 5.2 Representative HCMV viral load profiles in the blood of 6 liver transplant recipients with HCMV disease. The clinical course of the patients together with fluctuations in ALT levels, donor/recipient serostatus and administration of antiviral therapy are also indicated.

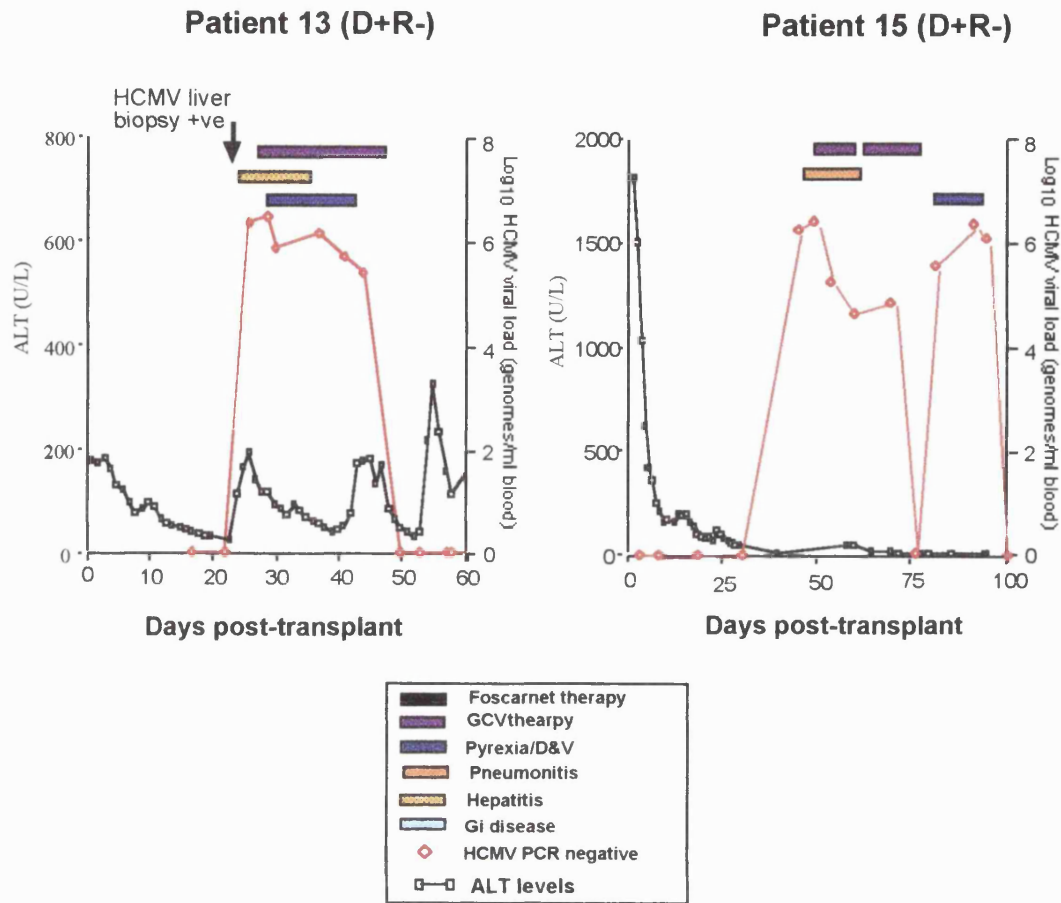


Figure 5.2 Continued.

5.3.3 Relationship between HCMV viral load and disease

The relationship between the maximum viral load and HCMV disease was analysed for the 47 liver transplant recipients subjected to HCMV DNA quantification. The scatter diagram shown in figure 5.3 shows the distribution of maximum viral loads in the patients with and without HCMV disease, where the solid line represents the median maximum viral load for each group. The median maximum viral load during follow-up in the 20 patients who experienced HCMV disease was $10^{5.65}$ genomes/ml blood (range $10^{4.94}$ - $10^{7.57}$ genomes/ml blood) and was significantly higher than in the 27 asymptomatic patients (median= $10^{4.29}$ genomes/ml blood, range $10^{3.59}$ - $10^{5.3}$; $p < 0.0001$, Mann-Whitney U test). This difference in viral load corresponds to 1.36 \log_{10} genomes/ml blood or a 133-fold increase in patients with disease.

5.3.4 Relationship between HCMV viral load and donor/recipient serostatus

Similar analysis was performed to examine the relationship between HCMV disease, viral load in blood and the donor/recipient serostatus of the 47 liver transplant recipients. A scatter diagram of the maximum viral loads together with details of the HCMV serostatus of the patients are shown in figure 5.4. In the D+R- group of 17 patients with primary infection, 12 experienced HCMV disease (70.6%). The median viral load in this group (median= $10^{5.43}$ genomes/ml blood, range $10^{3.59}$ - $10^{7.57}$) was significantly higher than in D-R+ group of patients reactivating latent HCMV (median= $10^{4.0}$ genomes/ml blood, range $10^{3.66}$ - $10^{4.92}$; $n=11$; $p < 0.01$ Mann-Whitney U test) where only one patient (9.0%) suffered disease. In patients at risk of either reactivation or reinfection 7 patients suffered HCMV disease (36.8%). The median viral load in this group (median= $10^{4.94}$ genomes/ml blood; range, $10^{3.64}$ - $10^{7.53}$; $n=19$) was significantly higher than in the D-R+ group ($p < 0.05$; Mann-Whitney U test). There was no significant difference between the median viral load in the D+R- group and the D+R+ group. Thus, patients at risk of either reactivation or reinfection had an intermediate risk of HCMV disease. If the D+R+ group were further stratified according to the median viral load within this group (median= $10^{4.94}$ genomes/ml blood), the 9 patients with viral loads above the median were significantly more at risk of disease than the 12 patients with levels below the median virus load ($p=0.02$ Fisher's exact test). An alternative analysis of this D+R+ group assessing viral load in the context of HCMV disease showed symptomatic patients (median= $10^{5.57}$ genomes/ml, range $10^{4.94}$ - $10^{7.53}$; $n=7$) had a significantly higher viral load than asymptomatic patients (median= $10^{4.58}$ genomes/ml, range $10^{3.64}$ - $10^{5.30}$; $p < 0.01$, Mann-Whitney U test).

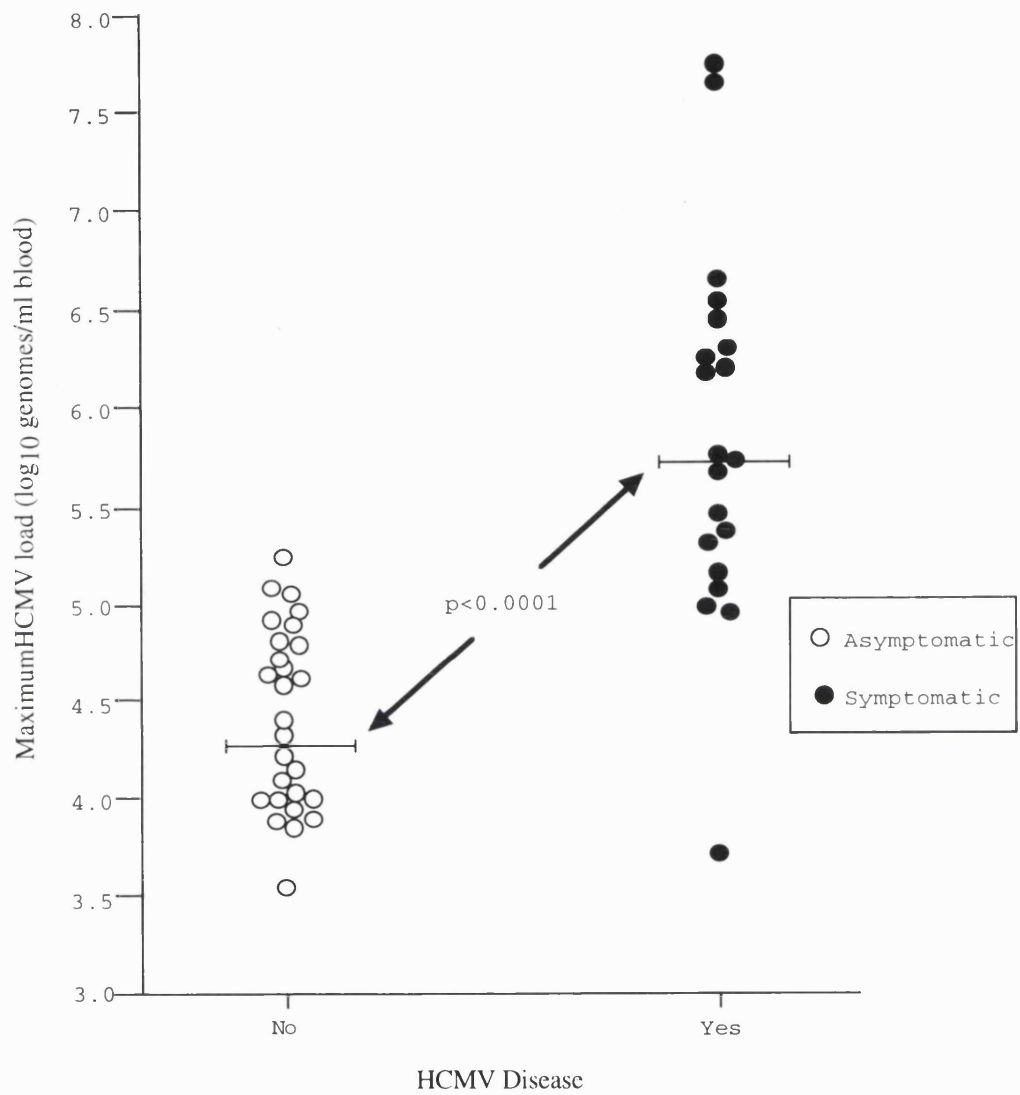


Figure 5.3 Relationship between maximum HCMV viral load in blood and disease in 47 liver transplant recipients.

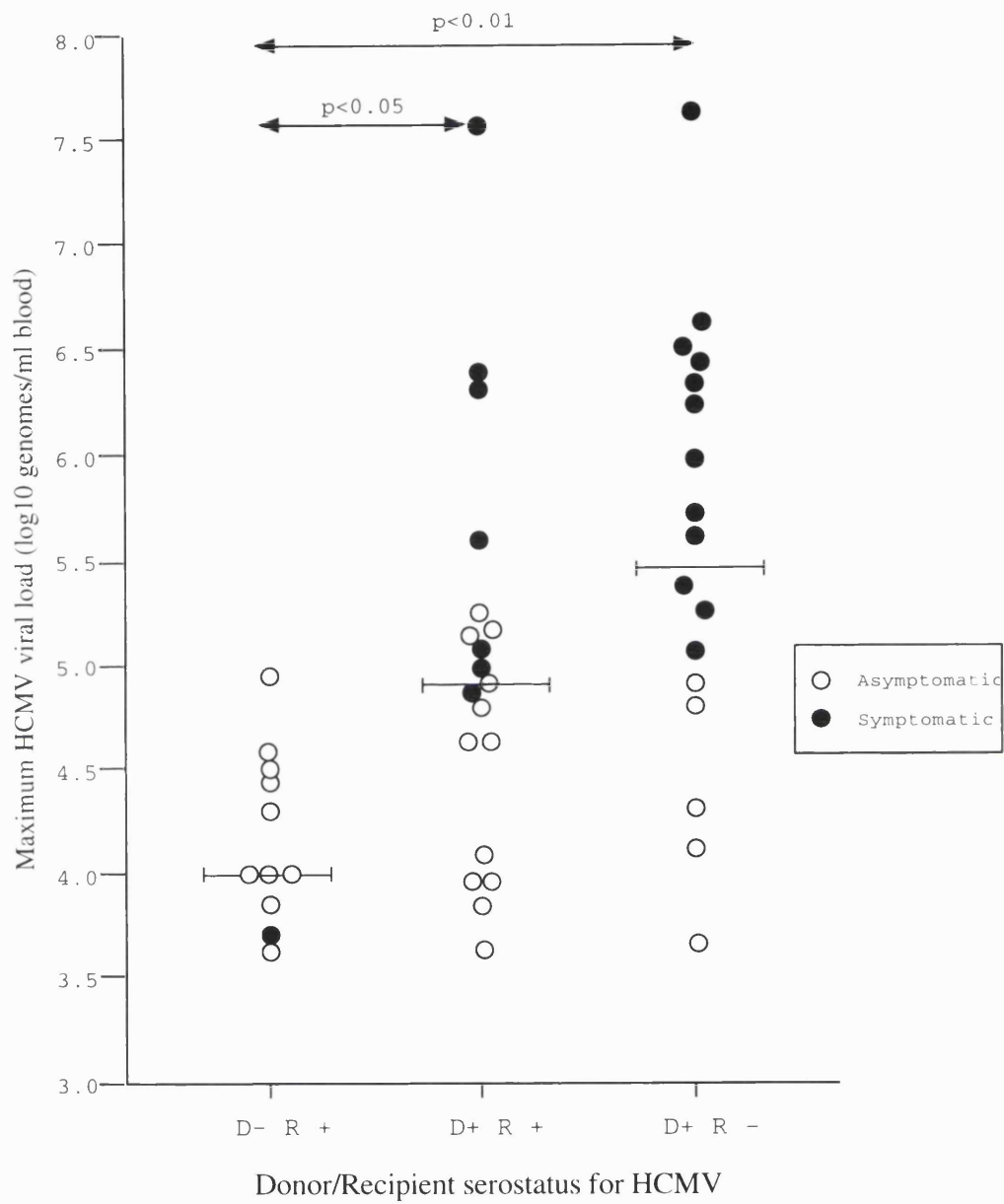


Figure 5.4 Relationship between maximum HCMV viral load, donor/recipient serostatus and HCMV disease in 47 liver transplant recipients.

5.3.5 Relationship between HCMV viral load and antiviral therapy in liver transplant recipients with HCMV disease

The clinical details from 20 liver transplant recipients who experienced HCMV disease are shown in table 5.2. Nine patients (45%) had persistent pyrexial debilitating disease, one patient had gastrointestinal disease (5%) confirmed by biopsy, six patients had pneumonitis (30%) and four patients (20%) had HCMV hepatitis. Sixteen patients received antiviral therapy which was given after the onset of symptoms, 15 patients were treated with ganciclovir therapy alone and one patient (patient 2) received both foscarnet and ganciclovir. The remaining four symptomatic patients (patients 4, 8, 18 & 20) were not treated, two of whom developed pneumonitis, one had hepatitis and one patient had persistent pyrexial debilitating disease.

All patients receiving ganciclovir experienced an overall reduction in viral load during administration of antiviral therapy as shown in figure 5.5. Three patients were excluded from this analysis due to either treatment with more than one antiviral agent (patient 2) or infrequent sampling (patient 12), one patient also experienced an increase in viral load during therapy, possibly due to the emergence of resistant strains (patient 10). In the remaining 13 patients 11 became HCMV PCR negative during ganciclovir therapy, where the mean reduction in viral load during therapy was 2.10 log₁₀ genomes/ml blood (median= 1.94 log₁₀ genomes/ml, range 0.81 - 3.98) when considering the pre-treatment viral load versus the first post treatment viral load. The changes in HCMV viral load after therapy had stopped are illustrated in figure 5.6. Eight patients (57%) remained PCR negative upon follow up, while six patients (43%) experienced a second episode of viraemia after stopping ganciclovir therapy (figure 5.7).

Table 5.2 Clinical characteristics from 20 liver transplant recipients with HCMV disease. HCV (hepatitis C virus), PSC (Primary sclerosing cholangitis), PBC (primary biliary sclerosis), FHF (fulminant hepatic failure), NANB (non-A non-B hepatitis virus), HCC (hepatocellular carcinoma).

Patient	Primary disease	D/R serostatus	Onset (days)	Symptoms & Biopsy (Bx) results	Antiviral Treatment & duration
1	HCV	D+R+	59	Pyrexia	GCV 28 days
2	Chronic rejection	D+R-	20	Pyrexia, leukopenia, bronchialveolar lavage (BAL) +ve	GCV 13 days, Fos 21 days
3	PSC	D+R-	35	Hepatitis, liver biopsy +ve	GCV 14 days
4	PBC	D+R+	90	Pneumonitis	none
5	Alcoholic cirrhosis	D+R+	126	Pyrexia, G.I disease, gastric biopsy +ve	GCV 21 days
6	HCV	D+R-	40	Pyrexia, diarrhoea	GCV 14 days
7	Glycogen storage	D+R-	32	Pyrexia, pneumonitis	GCV 14 days
8	Alcoholic cirrhosis	D+R-	60	Pyrexia	none
9	Alcoholic cirrhosis	D+R-	50	Pyrexia	GCV 17 days
10	Alcoholic cirrhosis	D+R+	40	Pyrexia	GCV 11 days
11	PBC	D+R-	35	Pneumonitis	GCV 14 days
12	Hep B/C/D	D+R-	43	Hepatitis, liver biopsy +ve	GCV 13 days
13	FHF NANB	D+R-	22	Pyrexia, hepatitis, liver biopsy +ve	GCV 26 days
14	HCV	D+R+	40	Pyrexia	GCV 7&15 days
15	PSC	D+R-	43	Pneumonitis, pyrexia, diarrhoea	GCV 10&14 days
16	Alcoholic cirrhosis	D+R+	65	Pyrexia	GCV 20 days
17	PSC	D+R-	34	Pyrexia	GCV 12 days
18	FHF	D+R+	31	Hepatitis, liver biopsy +ve	none
19	HCC	D+R-	60	Pneumonitis, liver biopsy +ve	GCV 36 days
20	PBC	D-R+	90	Pneumonitis	non e

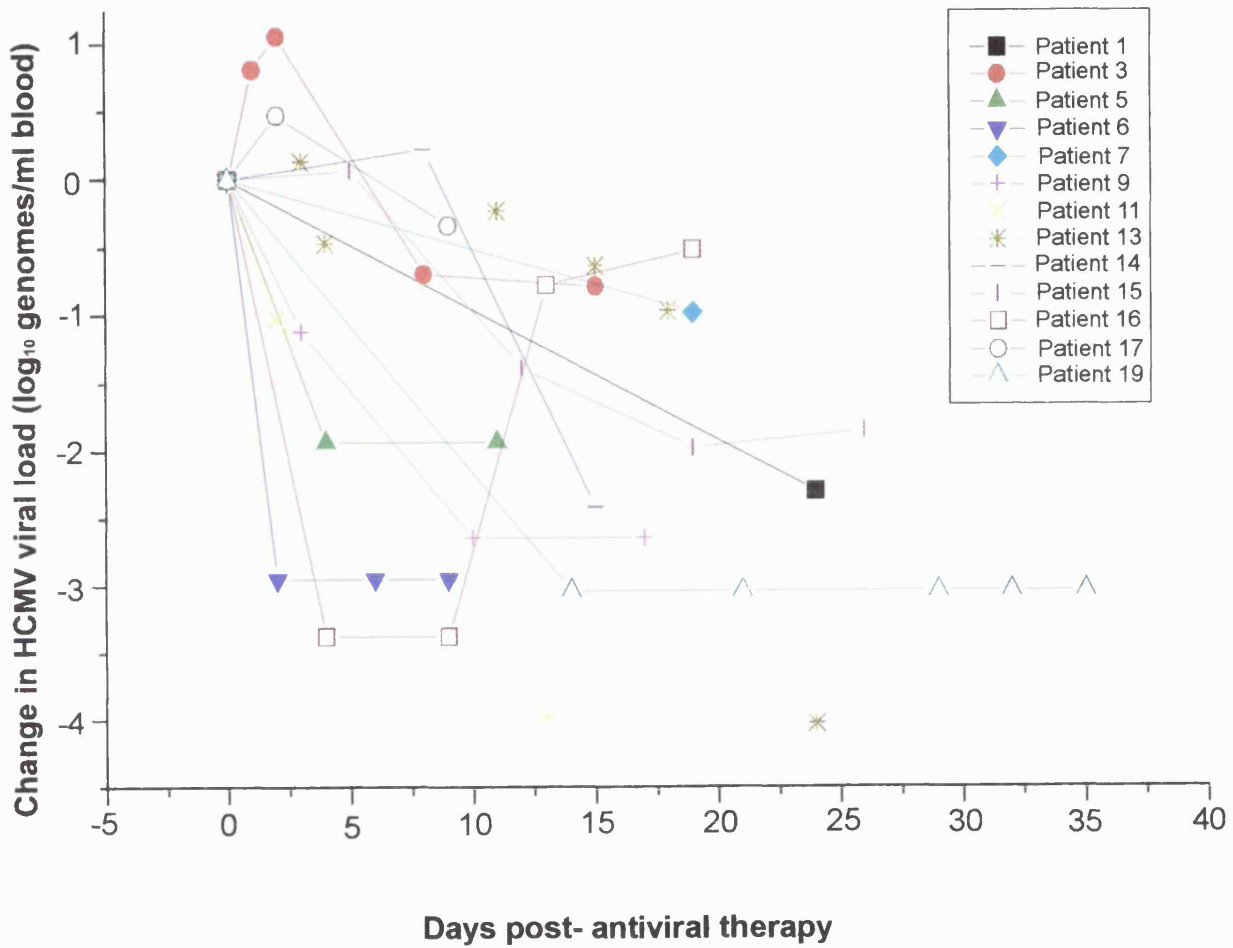


Figure 5.5 Log₁₀ change in HCMV viral load in blood in 13 liver transplant recipients receiving ganciclovir. Each line represents the change in load experienced by each patient during treatment with antiviral therapy.

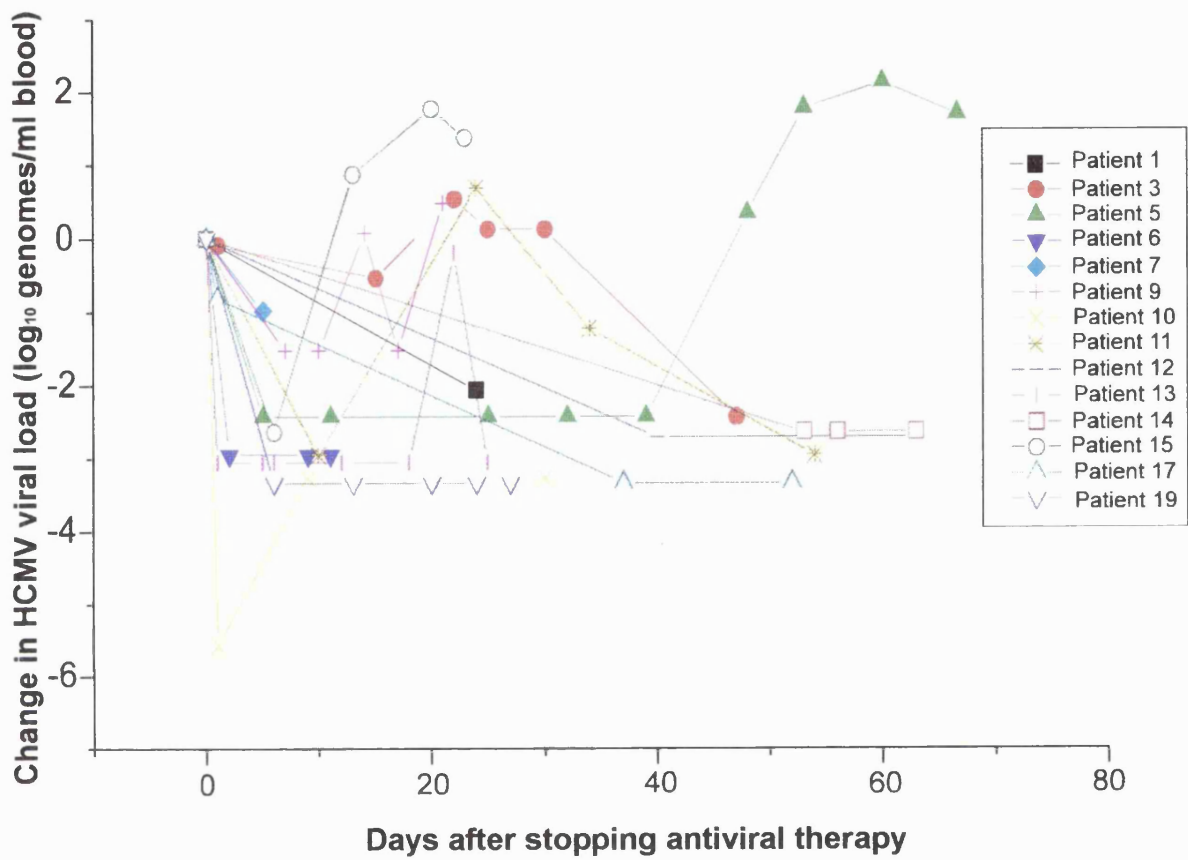


Figure 5.6 Log₁₀ change in HCMV viral load in 14 liver transplant recipients. Each line represents the change in load after cessation in ganciclovir therapy.

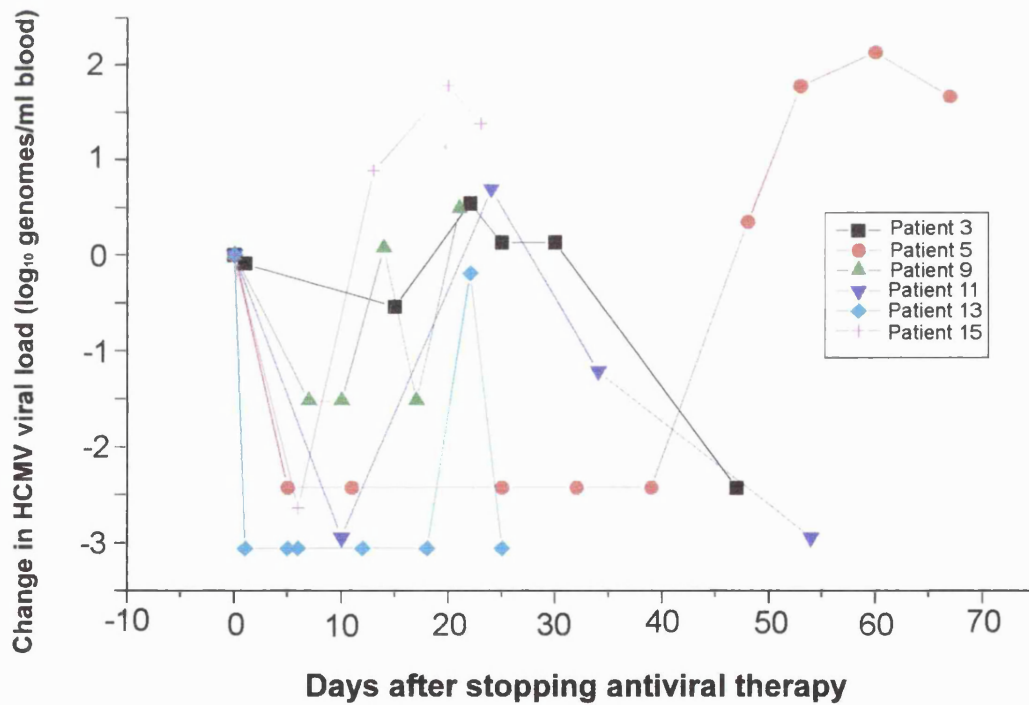
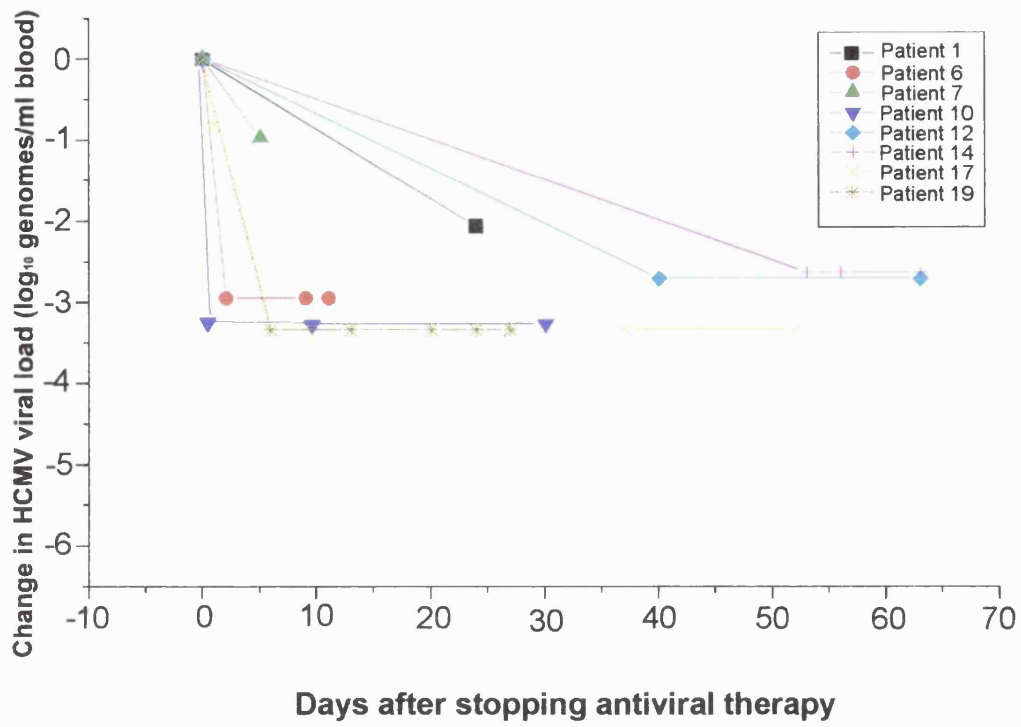


Figure 5.7 Changes in HCMV viral load after therapy had stopped. The top graph shows a sustained reduction in viral load, the bottom graph shows patients that experienced a resumption of viraemia.

5.3.6 HCMV viral clearance rates in liver transplant recipients receiving antiviral therapy

The effect of antiviral therapy on reducing HCMV viral load in the blood of liver transplant recipients was analysed further by calculating the viral clearance rates as described for congenitally infected infants in chapter 3 section 3.3.7. The estimated half-life ($t_{1/2}$) of the virus in blood was analysed in 13 of the 16 liver transplant recipients receiving antiviral therapy, patients 2, 10 and 12 were excluded from this analysis as mentioned in section 5.3.4. The clearance rates or $t_{1/2}$ for each patient are shown in table 5.3. The estimated clearance rates ranged from 0.41 and 5.56 days with a mean of 2.08 days (median= 2.55 days). There was no correlation between the pre-treatment HCMV viral load and the $t_{1/2}$ ($p=0.351$, $Rho=0.27$ Spearman's rank correlation). However, there was a correlation between the pre-treatment viral load and the first HCMV viral load after therapy had stopped ($p=0.0314$, $Rho= 0.62$, Spearman's rank correlation).

5.4 Quantification of risk factors for HCMV disease

5.4.1 Univariate statistical analysis

In order to assess the relative contributions of the risk factors for HCMV disease in liver transplant recipients, elevated viral load, donor/recipient seropositivity and the administration of augmented immunosuppression for rejection episodes were analysed in association with disease. Initially, the statistical analysis revealed that donor seropositivity was significantly associated with disease ($p=0.01$, Fisher's exact test) and there was a protective effect observed for recipient seropositivity ($p=0.006$, Fisher's exact test). The combinations of donor/recipient serostatus at baseline were analysed further by the Wilcoxon test in pairwise comparisons. There were significant associations between the following groups; D+R- versus D-R+ ($p=0.001$), D+R- versus D+R+ ($p=0.09$) and D-R+ versus D+R+ ($p=0.01$), indicating the importance of donor seropositivity as a risk factor for disease.

Elevated viral load, donor/recipient seropositivity and total augmented methylprednisolone were then quantified as risk factors for disease in a univariate model. The results of this analysis are shown in table 5.4. The administration of ATG, FK506 and OKT3 for rejection episodes were not significant risk factors for disease and hence were not analysed further. Significant associations were observed between increased viral load donor seropositivity, methylprednisolone administration and disease with odds ratios of 2.22 per 0.25 \log_{10} increase in viral load ($p=0.001$), 4.11 ($p=0.05$) and 1.30 per 1g increase in methylprednisolone ($p=0.01$) respectively.

Table 5.3 Estimated viral clearance rates ($t_{1/2}$) from 13 liver transplant recipients receiving ganciclovir therapy.

Patient	Log₁₀ genomes/ml before therapy	Log₁₀ genomes/ml after therapy stopped	$t_{1/2}$ (days)
1	4.36	2.30	3.51
3	6.48	4.64	1.96
5	4.24	2.30	0.78
6	5.25	2.30	0.41
7	5.43	4.45	5.53
9	4.96	3.83	1.87
11	6.28	2.30	1.36
13	6.33	5.36	5.56
14	4.73	2.30	2.60
15	6.54	4.65	4.47
16	5.68	2.30	0.44
17	5.64	4.83	2.61
19	5.34	2.30	2.07

Table 5.4 Univariate analysis relating the odds ratios for disease with viral load in blood, donor/recipient serostatus and receipt of methylprednisolone as risk factors for HCMV disease in liver transplant recipients.

Parameter	Odds Ratio (OR)	95% CI	p-value
Viral load (per 0.25 log ₁₀)	2.22	1.37 - 3.59	0.001
Donor/Recipient serostatus			
D+R-	4.11	1.02 - 16.67	0.05
D-R+	0.17	0.02 - 1.64	0.13
D+R+	1.0	-	-
Methylprednisolone (per 1g)	1.30	1.05 - 1.60	0.01

5.4.2 Multivariate statistical analysis

Multivariate analysis was performed on the significant risk factors identified in the univariate analysis in order to determine whether these variables were acting independently or were reliant on each other. The relative contributions of each risk factor was quantified using multiple logistic regression analysis and these data are shown in table 5.5. In the bivariate model, donor seropositivity remained a significant risk factor when analysed in the context of methylprednisolone, with little effect on the odds ratio (OR 4.80, $p=0.05$), however, this effect was negated after controlling for viral load (OR 3.24, $p=0.25$). The dominant effect of elevated viral load and disease remained a significant risk factor for disease after controlling for either donor/recipient serostatus or methylprednisolone (OR 2.15, $p=0.005$ and OR 2.70, $p=0.003$ respectively). Total methylprednisolone also remained a significant risk factor for disease after controlling for viral load with an odds ratio of 1.61 ($p=0.03$). In the case of recipient seropositivity, this remained non-significant when controlling for viral load increasing the odds ratio from 0.17 to 1.36 ($p=0.83$) observed in the univariate setting. Thus, the multivariate analysis identified both elevated viral load and total dose of methylprednisolone as independent risk factors for HCMV disease, whereas donor seropositivity was explained by elevated viral load.

5.4.3 Computed Probability of HCMV disease

On the basis of the logistic regression analysis a disease probability curve for increasing viral loads in the univariate setting was constructed and is shown in figure 5.8. The graph illustrates the expected probability of becoming symptomatic at any given viral load in the 47 liver transplant recipients studied. The viral load - disease probability curve shows that at low viral loads of between 10^4 and $10^{4.5}$ genomes/ml blood the probability of disease is also low (5-10%). However, once a viral load of $10^{4.75}$ genomes/ml blood is reached, the probability of becoming symptomatic rises dramatically reaching 80% at $10^{5.5}$ genomes/ml blood. A similar graph was produced from the multivariate analysis to investigate the effect of methylprednisolone dose on the probability of suffering HCMV disease with increasing viral loads (figure 5.9). Increasing amounts of total augmented methylprednisolone shifted the curve to the left such that considerably lower viral loads were required to cause disease. For example when 3g methylprednisolone was administered, a viral load of $10^{4.75}$ genomes/ml gave a disease probability of 10%, however at the same viral load in patients receiving 12g methylprednisolone a disease probability of 90% was reached.

Table 5.5 Multivariate logistic regression analysis to examine pairs of risk factors on the odds ratios for HCMV disease in liver transplant recipients obtained from the univariate model.

Parameter	Odds Ratio (OR)	95% CI	p-value
Viral load and D/R serostatus			
Viral load (per 0.25 log ₁₀)	2.15	1.26 - 3.66	0.005
D+R-	3.24	0.43 - 24.33	0.25
D-R+	1.36	0.09 - 21.58	0.83
D+R+	1.0	-	-
Methylprednisolone and D/R serostatus			
D+R-	4.80	0.98 - 23.51	0.05
D-R+	0.07	0.004 - 1.29	0.07
D+R+	1	-	-
Viral load and Methyprednisolone			
Viral load (per 0.25 log ₁₀)	2.70	1.41 - 5.17	0.003
Prednisolone (per 1g)	1.61	1.04 - 2.51	0.03

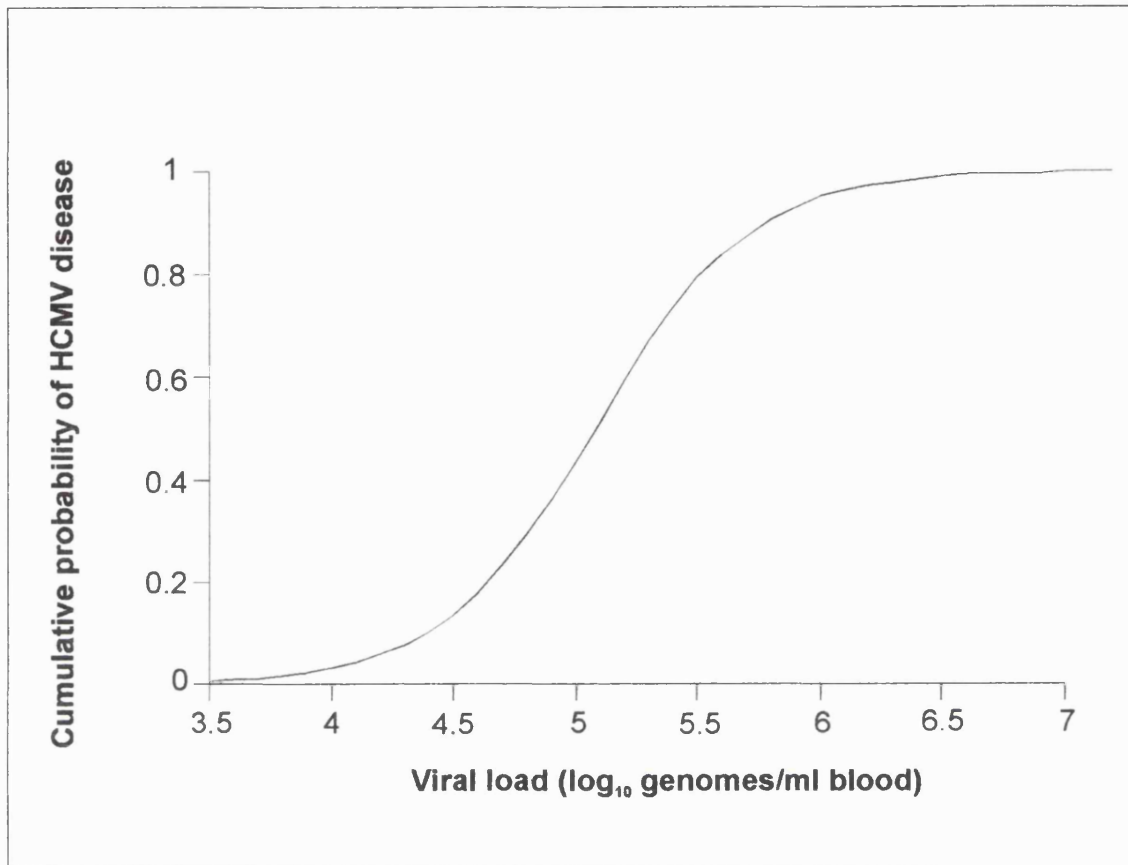


Figure 5.8 Probability of HCMV disease with increasing viral load calculated from the univariate regression analysis

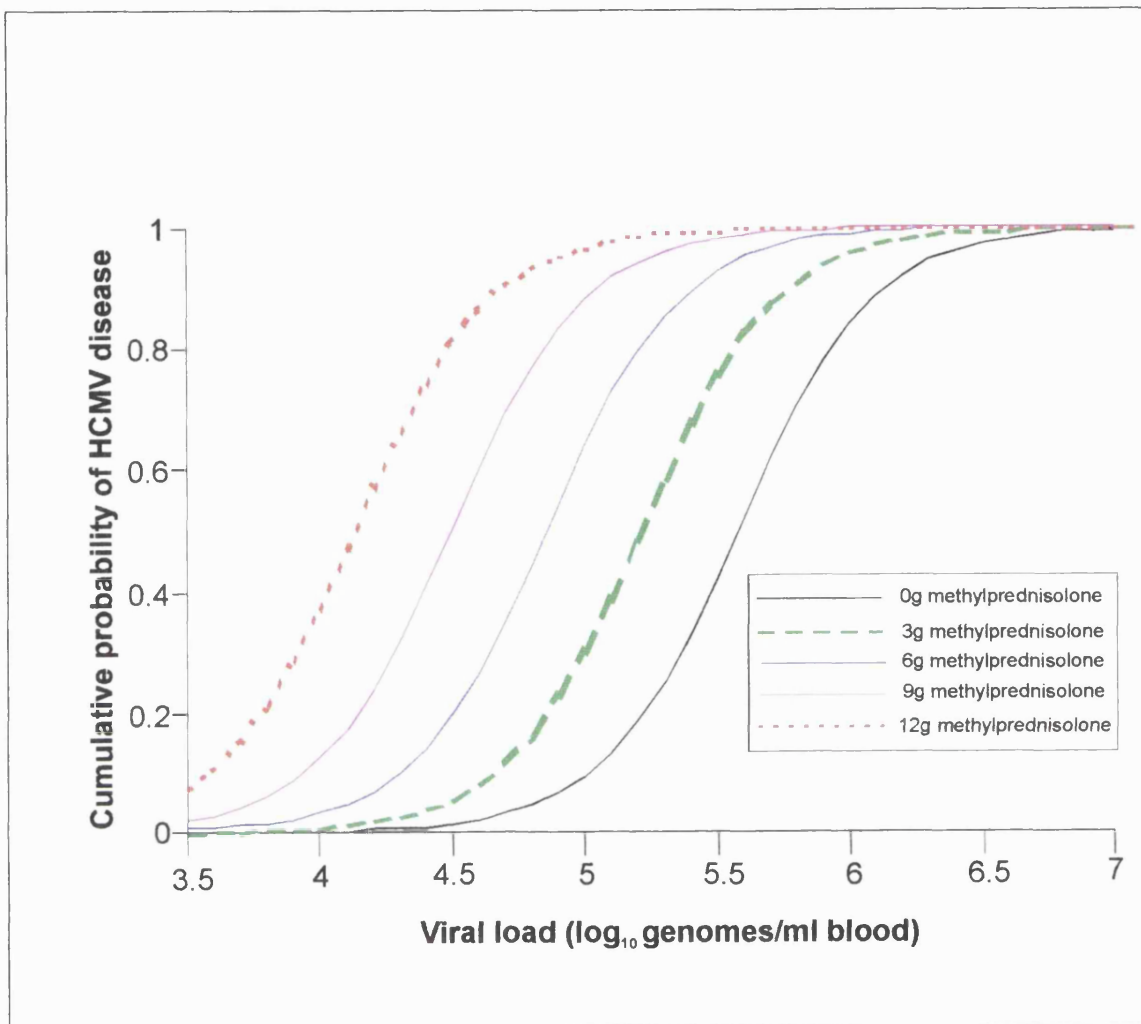


Figure 5.9 Probability of HCMV disease with increasing viral load and methylprednisolone calculated from the multivariate logistic regression analysis.

5.5 Discussion

The results presented in this chapter clearly indicate that increased HCMV viral load in blood is a major risk factor for disease in liver transplant patients. As observed for the urine in renal transplant recipients described in chapter 4 of this thesis, patients experiencing HCMV disease had a consistently higher median viral load compared with patients who remained asymptomatic ($p < 0.0001$). The median peak viral load was also significantly higher in those patients undergoing primary infection, 70.6% of whom developed disease compared to patients reactivating latent HCMV only 9.0% of whom developed disease ($p < 0.01$). The univariate analysis identified the administration of immunosuppressive therapy and donor seropositivity as risk factors for disease which is consistent with several other studies (Badely *et al*, 1996, Sutherland *et al*, 1992, Portela *et al*, 1995, Lao *et al*, 1997, Falagas *et al*, 1996). In addition, elevated viral load was identified as a significant risk factor for disease in liver transplant recipients which has not been previously described in a detailed longitudinal study.

Donor serostatus as a significant risk factor for disease was unaffected by controlling for methylprednisolone administration, but was negated after controlling for viral load in the multivariate logistic regression analysis. This result is similar to that observed for donor/recipient serostatus in renal transplant recipients detailed in chapter 4. Thus, it can be assumed that viral load is the mechanism through which this risk factor is associated with disease ie. patients with primary infection have HCMV disease due to higher viral loads. The longitudinal viral load analysis undertaken in this chapter also provides insight into the pathogenesis of HCMV disease. In patients at risk of either reactivation or re-infection (D+R+ group) an intermediate risk of disease was noted when compared to those patients with primary infection or reactivation of latent HCMV as was observed for renal transplant recipients discussed in chapter 4. Patients in this group with viral load levels above the median were associated with increased risk of disease and presumably were more likely to be experiencing primary infection of exogenous virus, while those below the median were reactivating their own virus and had a lower risk of disease. To confirm this suggestion, restriction fragment length polymorphism studies of larger numbers of patients in this category will be required. In contrast to donor/recipient seropositivity, methylprednisolone and elevated viral load were independent risk factors for disease where their significance remained constant in the multivariate analysis ($p = 0.03$, $p = 0.003$, respectively). This observation implies that receipt of large quantities of methylprednisolone for rejection episodes predisposes the individual to HCMV disease at lower viral load levels. In contrast, higher viral loads may be necessary to produce

pathologic consequences in patients who experience relatively few rejection episodes and therefore require lower levels of augmented methylprednisolone.

The effect of antiviral therapy on reducing HCMV viral load was analysed in patients receiving ganciclovir therapy. There was a sustained effect of ganciclovir therapy on HCMV viral replication since 57% of patients remained PCR negative after therapy had stopped. The effect of ganciclovir therapy was analysed further by calculating the estimated clearance rates, the mean $t_{1/2}$ of HCMV in the blood was 2.55 days. There was a significant correlation between the pre-treatment viral load and the first viral load after therapy had stopped. In contrast, there was no correlation between the pre-treatment viral load and the half-life of decline of the virus in blood. This was consistent with similar analysis of urine in congenitally infected infants detailed in chapter 3. These data suggest that the ability to clear systemic HCMV may be controlled by factors other than the quantity of virus in the blood and are likely to be influenced by the immune status of the individual. Unfortunately due to small numbers of patients, the relationship between $t_{1/2}$, the donor/recipient serostatus and the administration of augmented immunosuppression could not be analysed from this data set. Further studies with larger groups of patients investigating these parameters may elucidate to the factors involved in the clearance of HCMV.

The disease probability-viral load curves computed from the logistic regression analysis support the interpretation that HCMV disease is imminent once specific viral load levels are reached. Thus, a 50% probability of disease was reached at a viral load of $10^{5.1}$ genomes/ml blood and a 90% probability of disease was reached at $10^{5.5}$ genomes/ml blood without consideration of methylprednisolone dose. The influence of increasing amounts of methylprednisolone on the viral load-disease probability curve was to shift the curves to lower viral loads. Hence, at a methylprednisolone level of 12 g the 50% disease probability level was reached at a viral load of $10^{4.1}$ genomes/ml blood ie a 10-fold lower peak virus load than in patients receiving no methylprednisolone. This result supports the clinical practice of reducing steroid therapy, whenever possible, in the cases of HCMV infection post-transplant. Further analysis on more patients will elucidate whether the temporal increase in viral load can be used to direct pre-emptive therapy in those patients most at risk of HCMV disease and suggests that controlled trials of such interventions should stratify patients according to dose of methylprednisolone received.

Various strategies have attempted to identify and reduce disease incidence in patients

at risk of HCMV disease by applying different treatment regimens, with varying success (Portela *et al*, 1995, Paya *et al*, 1993, Van Den Berg *et al*, 1993, Singh *et al*, 1994, Winston *et al*, 1995b, Snyderman *et al*, 1994, Stratta *et al*, 1992, Cohen *et al*, 1993, Mollison *et al*, 1993) . Prophylaxis, although effective in the reduction of HCMV disease, also exposes patients not destined to develop disease to potentially toxic antiviral drugs. Thus, the need to find a reliable marker for disease is of paramount importance. In this chapter, it was possible to identify those individuals most at risk for disease by using a combination of quantitative PCR and the administration of methylprednisolone. The use of viral load increases above critical levels to identify patients at risk of future disease is an attractive option, but further studies are required to ascertain whether such an approach would be applicable in the clinical setting.

In conclusion, the detailed analysis of patients by measurement of viral load and frequent sampling may offer the possibility of designing algorithms which will predict whether a patient will go on to develop HCMV disease. Such monitoring, together with the knowledge of other risk factors such as methylprednisolone usage, may identify not only patients at risk of disease but also will help prevent patients receiving unnecessary treatment with potent antiviral therapy.

Chapter 6.

Correlation between longitudinal changes in HCMV load, total HCMV IgG and anti-gB antibodies following liver transplantation.

6.1 Introduction

The role of antibodies directed towards HCMV and their ability to control infection and disease has been the topic of much discussion for many years. Although it is clear that the cellular immune response is necessary for the resolution of viral infections, the humoral immune response has also been implicated in the control of infection and disease. Numerous polypeptides of HCMV have been identified that are immunogenic, the most abundant antibodies directed to HCMV are the phosphoproteins pp150, and pp65, however, these antibodies are not capable of neutralising the virus. Antibodies directed to the envelope glycoproteins are of particular importance since they are potentially neutralising and have the ability to eliminate virus infected cells. The majority of neutralising antibodies are directed against glycoprotein B of HCMV. Glycoprotein B (gp58 -116), the most abundant envelope protein, consists of a complex of two components linked together by disulphide bonds; gp116 at the amino terminus and gp58 at the carboxy terminus (see chapter 1 section 1.1.5.3, figure 1.4). Most of the neutralising antibodies are targeted towards the gp58 portion of the molecule, accounting for 70% of the total neutralising capacity in human sera (Cranage *et al*, 1986, Britt *et al*, 1990, Kniess *et al*, 1991, Marshall *et al*, 1992). Further characterisation of gp58 has led to the identification of two major linear neutralising epitopes, AD-1 and AD-2, of which AD-1 appears to be the most immunogenic with IgG1 sub-class reactivity (Kneiss *et al*, 1991, Urban *et al*, 1994, Schoppel *et al*, 1996).

Antibodies towards gB are produced during natural infection with HCMV in both immunocompetent and immunocompromised individuals, the extent of which appears to be highly variable (Reddehase *et al*, 1991, Wang *et al*, 1996, Schoppel *et al*, 1997, Navarro *et al*, 1997). Several studies have attempted to elucidate the importance of these antibodies in the control of infection with particular emphasis on the use of the murine model (Reddehase *et al*, 1994, Jonjic *et al*, 1994). One study by Reddehase *et al* (1994) examined viral recurrence from latency in neonatal mice with primary infection compared to mice infected during adult life. Mice infected in the neonatal period suffered a 40% mortality rate due to recurrent infection despite high titres of neutralising antibody. However, neutralising antibodies were responsible for limiting virus dissemination to multiple organs, since latently infected mice suffered discrete organ involvement. In contrast, non-primed mice suffered multiple organ involvement upon super-infection at high dose viral challenge. Indeed, the role of antibodies appeared to be of central importance in controlling virus dissemination in primed mice but was insignificant at controlling infection in mice with primary infection. The kinetics of viral

clearance was comparable between B-cell deficient mice and those with an intact immune system during primary infection. However, viral titre in various organs was higher in B-cell deficient mice upon recurrence. The administration of immune sera to these mice resulted in a 2 log₁₀ reduction in viral titre in the spleen and a 1.5 log₀ reduction in the lungs, indicating the central role of these antiviral antibodies at reducing viral dissemination in multiple organs (Jonjic *et al*, 1994).

The role of the passive transfer of antiviral antibodies to the fetus during pregnancy may be crucial in protection from congenital infection. Several studies have reported a reduced incidence of HCMV infection in infants born to pre-immune mothers, compared to mothers undergoing a primary infection during pregnancy (Stagno *et al* , 1982, Fowler *et al* , 1992, Adler *et al*, 1995, Boppana *et al* , 1995 & 1996). Adler *et al* (1995) observed a significantly reduced risk of HCMV infection in seropositive women of child bearing age at risk of infection from children shedding virus in either urine or saliva. Similar findings were observed by Fowler *et al* (1992), where infants born to seropositive mothers had a significantly reduced incidence and severity of congenital HCMV infection. On the other hand, during primary maternal infection, the transfer of antiviral antibodies did not provide protection. In studies by Boppana *et al* (1995 & 1996) women undergoing primary HCMV infection produced high titres of neutralising antibodies to gB which did not reduce transmission *in vivo* or affect clinical outcome. Indeed, mothers who transmitted virus to the fetus had significantly higher titres of neutralising antibodies to gB, presumably reflecting the extent of virus replication.

Pass *et al* (1983) compared antibody responses between renal transplant recipients undergoing primary and recurrent HCMV infection. This early study suggested that circulating antibodies may have a protective effect against HCMV infection. Patients with primary infection experienced a delay in neutralising antibodies compared to patients with recurrent infection. Later Schoppel *et al* (1997) examined the immune response to both non-neutralising phosphoproteins and neutralising glycoproteins in immunocompetent and immunocompromised individuals. Immunocompetent seropositive individuals produced a qualitatively stable characteristic antibody profile predominantly to pp150 and the AD-1 domain of gB with only minor fluctuations in antibody titres over time. However, in immunocompromised patients following primary infection, there was a rapid increase in antibodies to the phosphoproteins pp65 and pp150 and a delayed response in antibodies to the neutralising glycoproteins. In contrast, patients with a primed immune response produced antibodies to non-neutralising and neutralising antigens both rapidly

and simultaneously following the detection of HCMV viraemia by PCR.

The administration of gB sub-unit vaccines and their ability to induce a sustainable immune response against HCMV in animal models has been established (Britt *et al* , 1995, Harrison *et al* , 1995). The use of live vaccines, however, for the prevention of HCMV disease in women of child bearing age is somewhat controversial. Currently, one trial exists for the use of such a vaccine based on the replication competent Towne strain of HCMV although the immunogenicity and safety of this vaccine is still in question. Wang *et al* (1996) examined the production of mucosal antibodies to HCMV gB in immunocompetent seronegative individuals receiving either live attenuated Towne strain vaccine or a purified recombinant gB sub-unit vaccine. The results from this study demonstrated that vaccinees were able to generate a sustained IgG response to gB in sera and mucosa, where the neutralising titres of serum antibodies to gB correlated with antibody levels to gB in mucosa. Recipients of the recombinant vaccine produced an IgG response to gB more rapidly and 7/10 vaccinees had a detectable soluble IgA response in saliva. The induction of an immune response at the mucosal membrane may be crucial when considering a vaccine for the protection of HCMV in seronegative pregnant women, since the most common route of transmission is via the oral mucosal route from children shedding virus in urine and or saliva. Further studies to establish the level and diversity of the humoral immune response are required before the evaluation of vaccines based on gB can be made. The detailed analysis of the kinetics of neutralising antibody response in the context of HCMV infection in the immunocompromised host will also assist the design of a suitable gB sub-unit vaccine. The following chapter describes the kinetics of antibody levels to gB and total HCMV IgG response in relation to the modulations in HCMV viral load following liver transplantation.

6.2 Patients Studied

6.2.1 Study population

The same patients described previously in chapter 5 of this thesis were also used for this prospective study. Briefly, a total of 162 liver transplant recipients with a total of 1,433 surveillance blood samples between November 1992 and April 1996 were analysed. Fifty one patients were HCMV viraemic, 47 of these were subjected to detailed quantitative HCMV PCR analysis. 127 blood samples were HCMV PCR positive and subjected to quantitation from a total of 467 samples analysed. Pre-transplant serum samples from donors and recipients were tested for the presence of HCMV IgG antibodies using a commercially available enzyme immunoassay (Biokit SA, Barcelona, Spain).

Sequential serum samples were collected from patients on a weekly basis and where clinically indicated. Sera were analysed for the presence of antibodies to gB using immunofluorescence in the IFA assay by end point dilution as described in chapter 2 sections 2.6.4 - 2.6.6. The same sera were also analysed for total HCMV IgG levels using a semiquantitative microparticulate enzyme immunoassay (AXSYM) analysis was performed by Abbott laboratories (Chicago Illinois, USA). For the purpose of this study, seven patients were excluded since only one post-transplant serum sample from each patient was available for analysis during surveillance. Of the remaining 40 patients analysed in detail, 50% experienced HCMV disease and 50% remained asymptomatic during the surveillance period. Seventeen patients (42.5%) were at risk of primary infection (D+R-), 58.8% of whom developed HCMV disease. 14 patients (35%) were at risk of either reactivation or reinfection (D+R+) 50% of whom developed HCMV disease compared to only one (11.1%) of the nine patients with reactivation of latent HCMV (D-R+). The median number of days of follow-up was 124.5 days post transplant (range 15 - 539) which was comparable between the D+R- (median=139 days, range, 119 - 539; n=17), D+R+ (median=109 days, range, 15 - 292; n=14) and D-R+ (median=124 days, range 31 - 338; n=9). The median number of days follow-up was significantly lower in asymptomatic patients (median = 90 days; range, 15 - 372 days) than in symptomatic patients (median = 166.5 days, range 61 - 539; p<0.05 Mann-Whitney U test). However, in all but one of the 20 symptomatic patients where the median time to the development of disease was 41.5 days (range 10 - 126 days) the onset of symptoms occurred prior to the median follow up time of 90 days observed for the asymptomatic group.

6.2.1.1 Qualitative detection of HCMV DNA

A total of 467 blood samples from 40 patients were screened qualitatively for the presence of HCMV DNA. 27% (127) of blood samples were positive by qualitative PCR for HCMV DNA and were subjected to quantitative analysis. The median number of blood samples analysed was 10 (range 3 - 24) which was comparable between the three donor/recipient groups. The median number of PCR positive samples in the 40 patient cohort was 2 (range 1- 11), which was significantly higher in the D+R- group (median= 3, range 1 - 11) compared to HCMV seropositive patients (median= 1, range 1 - 5 ; $p=0.03$, Mann - Whitney U test). The number of PCR positive samples was also significantly higher in patients with HCMV disease (median=3.5, range 1 - 11) compared to asymptomatic patients (median=1, range 1 - 4, $p=0.0002$ Mann-Whitney U test).

6.2.2 Immunosuppressive therapy

The triple therapy regimen for liver transplant recipients is described in detail in chapter 5 , section 5.2.2. All patients received triple therapy initiated immediately following transplantation which was adjusted according to renal function and plasma levels where necessary.

6.2.3 Antiviral therapy and antimicrobial chemotherapy

The details of antimicrobial therapy for prevention of bacterial and fungal infections are described in chapter 5, section 5.2.3. Antiviral therapy was only administered to patients at the onset of defined HCMV disease. Sixteen patients of the 40 patient cohort were treated with antiviral therapy, 15 patients received ganciclovir alone and one patient was treated with ganciclovir followed by foscarnet at the recommended doses.

6.3 Results

6.3.1 Qualitative detection of antibodies to HCMV gB in sequential serum samples

Four hundred and twenty three serum samples from the 40 patient cohort were analysed (median= 9.5; range 3 - 24) for anti-gB antibody levels, 400 (95%) of these were also analysed for the level of total HCMV IgG response. The median number of serum samples was comparable between HCMV seronegative recipients and HCMV seropositive recipients (median=10, range 3 - 24; median=8, range 3 - 24; respectively: $p>0.05$ Mann-Whitney U test). However, significantly more serum samples were collected from patients with HCMV disease compared to patients that remained asymptomatic, since samples were taken where clinically indicated for liver function tests (median= 13.5, range 3 - 24; median= 7.5, range 3 - 15; respectively: $p<0.0.1$, Mann - Whitney U test). A total of 50 (12.5%) serum samples from 12 patients were negative for antibodies to gB at a 1:30 dilution by IFA. Forty eight of these samples were from 11 patients with primary HCMV infection compared to 2 pre-transplant serum samples from a D-R+ patient which may have been detected if the IFA assay was performed on neat sera. The remaining 350 serum samples were all positive for anti-gB antibodies at least at a 1:30 dilution or greater.

6.3.2 Relationship between HCMV total IgG response and detection of HCMV DNAemia

The relationship between the level of total HCMV IgG response to the first episode of HCMV DNAemia ie the first detection of HCMV in blood by qualitative PCR (as described in chapter 2 section 2.9) and peak viral load was analysed in 32 liver transplant recipients. Eight patients were excluded from this analysis due to infrequent sampling preventing time dependent analysis. The initial IgG response was detected at a median time of 21 days post-transplant (range, 0 - 47 days), which was not significantly earlier than a median of 35.5 days for the first detection of HCMV DNAemia (range, 18 - 54 days) in patients with primary HCMV infection. The first IgG response was detected prior to the first episode of HCMV DNAemia in 26 patients and after the first detection of HCMV DNAemia in 6 patients. The initial HCMV total IgG response was detected at a median of 35 days (range, -269 days - +10 days) prior to the first detection of HCMV DNAemia in recipient seropositive patients, which was significantly earlier than the median of 12 days prior to the first detection of HCMV DNA observed in recipient seronegative patients (range, -49 - +12 days, $p<0.05$, Mann-Whitney U test, figure 6.1). Only one of the recipient seropositive patients had an initial total HCMV IgG response

after the first detection of HCMV DNAemia. In contrast to the detection of the initial total HCMV IgG response, the detection of the first episode of DNAemia was not detected significantly earlier following transplantation in HCMV recipient seropositive patients.

Peak total HCMV IgG was detected at a median of 42 days (range, 0 - 232 days) post-transplant, which was not significantly earlier than the detection of peak HCMV viral load (median=45.5 days; range 0 - 331 days). In recipient seropositive patients, peak total HCMV IgG levels appeared prior to peak viral load in 12 patients, at the same time in 4 patients and after peak viral load in one patient. In HCMV seronegative recipients, peak HCMV total IgG levels were detected after peak HCMV viral load in 11 patients, at the same time in 2 patients and prior to peak viral load in only one patient. Figure 6.2 shows the significant relationship between the peak HCMV viral load and peak total IgG response in HCMV seropositive and HCMV seronegative recipients. Recipient seropositive patients developed a peak IgG response at a median of 14.5 days (range, -258 - 28 days) prior to peak viral load, which was significantly earlier than seronegative recipients where peak total IgG was observed at a median of 40.5 days (range, 0 - 197 days; $p < 0.01$, Mann - Whitney U test) after the detection of peak viral load. The time of detection of initial and peak total HCMV IgG as well as the detection of initial DNAemia and peak HCMV viral load were not significantly associated with the HCMV disease status of the patients.

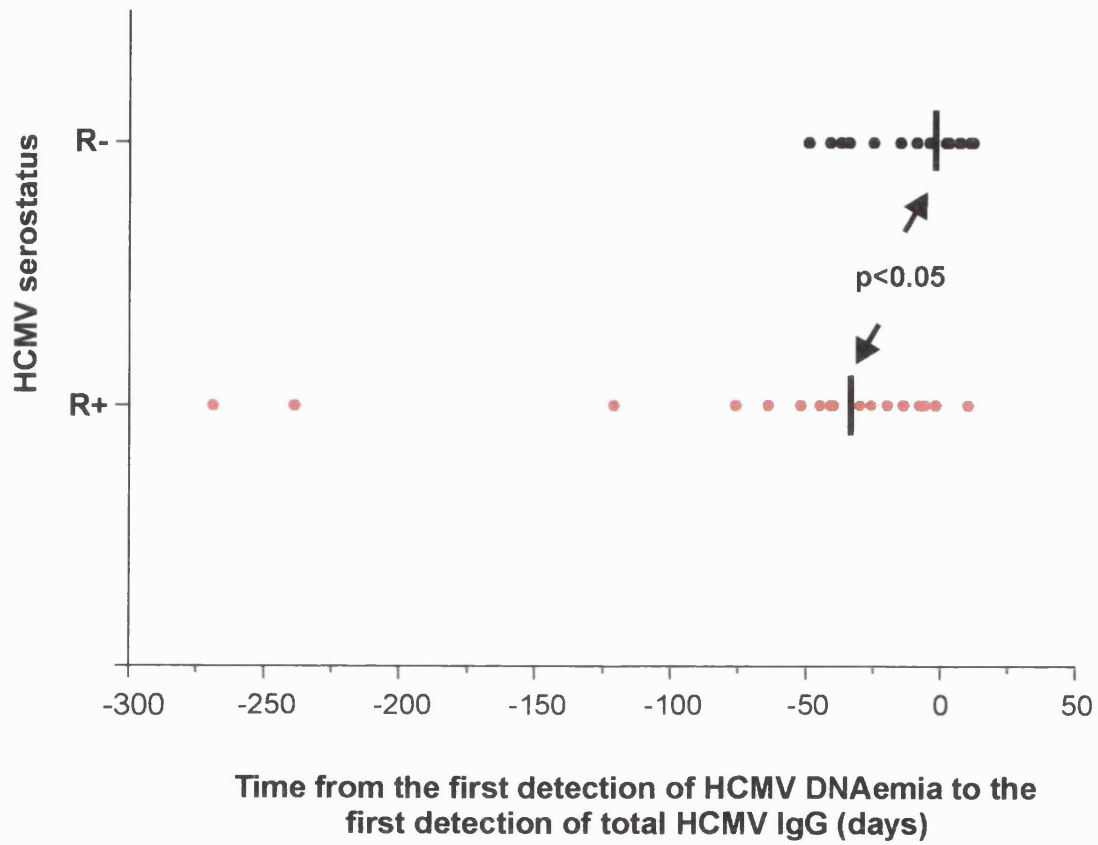


Figure 6.1 Relationship between HCMV serostatus and the time from the first detection of HCMV DNAemia to the first detection of an initial IgG response in 32 liver transplant recipients. The solid lines indicate medians for each group (Mann-Whitney U test). In this analysis, the detection of DNAemia was assigned as day 0.

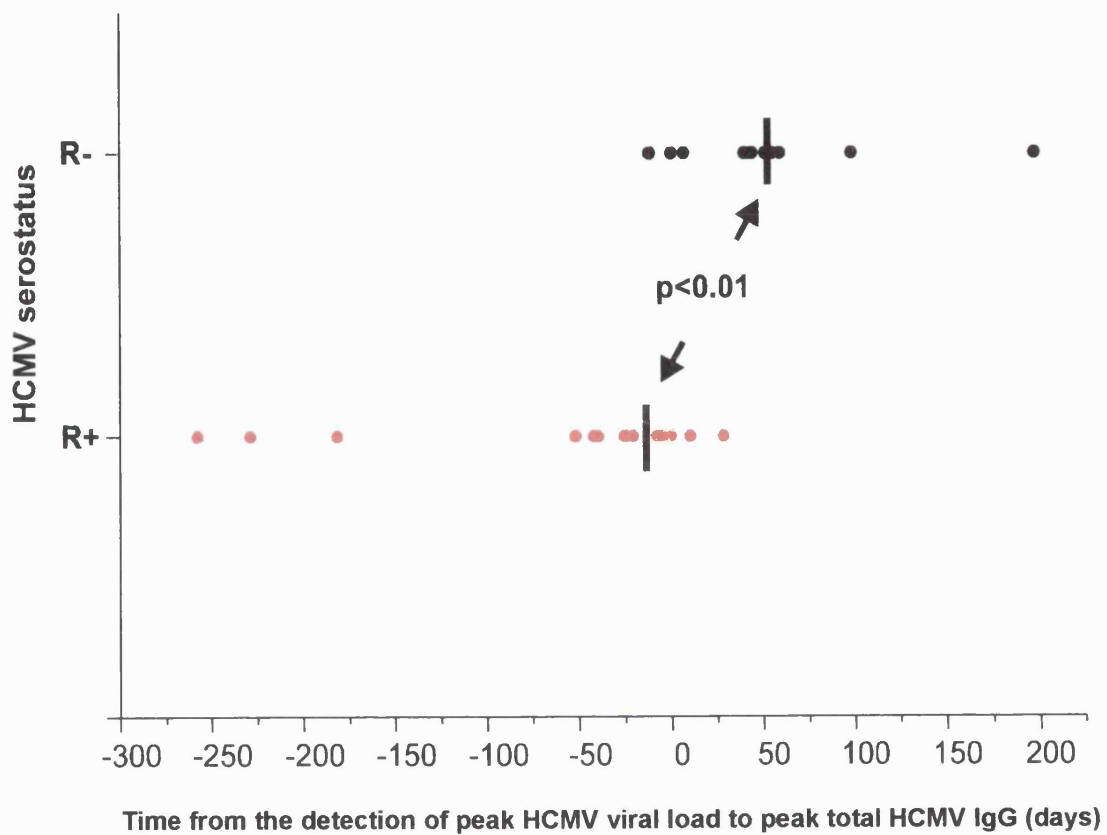
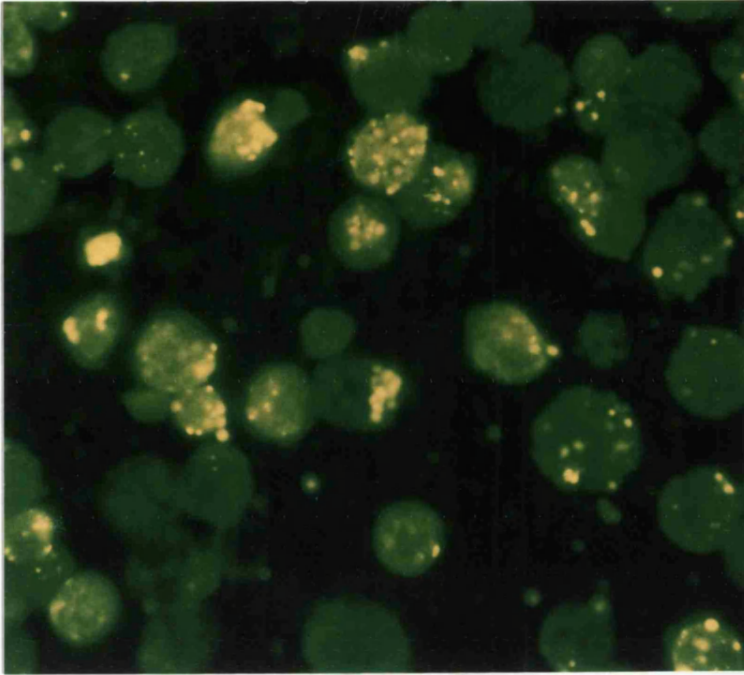


Figure 6.2 Relationship between HCMV serostatus and the time from peak HCMV viral load to peak total IgG response in 32 liver transplant recipients. The solid lines indicate medians for each group (Mann-Whitney U test). In this analysis, the detection of peak HCMV viral load was taken as day 0.

6.3.3 Relationship between time to first detection of HCMV DNAemia and first detection of a significant rise in anti-gB antibodies post-transplant

Thirty two patients were subjected to detailed analysis for the time to first detection of HCMV DNAemia, and time to the first detection of a significant rise in anti-gB antibodies post-transplant. A significant rise in anti-gB antibody level was defined as a minimum four-fold increase in end-point titre from the baseline detection of a 1:30 dilution of the sera (i.e a minimum detection at a 1:480 dilution). An example of the serial end point dilution of sera and detection by immunofluorescence is shown in figure 6.3. The detection of HCMV DNA by PCR appeared earlier than an initial rise in antibody levels to gB in 20 patients, at the same time as DNAemia in 7 patients and appeared later in 5 patients (figure 6.4) . Figure 6.5 shows the highly significant correlation between the first detection of HCMV DNA and first rise in anti-gB antibody levels following liver transplantation ($R=0.66$, $p=0.0003$, Spearman's rank correlation coefficient). The median number of days for the first detection of HCMV DNA was 37.5 days post-transplant (range 0 - 331) compared to a median of 46 days post-transplant (range 9 - 331 days) for a significant rise in anti-gB antibodies. Although this did not reach statistical significance ($p=0.08$, Mann - Whitney U test), there was a trend for the detection of HCMV DNAemia by PCR to precede a significant rise in gB antibody levels irrespective of HCMV serostatus. The first detection of a rise in anti-gB antibody levels was unrelated to the initial viral load in blood and did not appear significantly earlier post-transplant in the 19 symptomatic patients compared to the 13 asymptomatic patients. Similarly, there was no significant association between HCMV serostatus, timing to first rise in gB antibody levels or timing to first detection of HCMV DNAemia following transplantation.

a)



b)

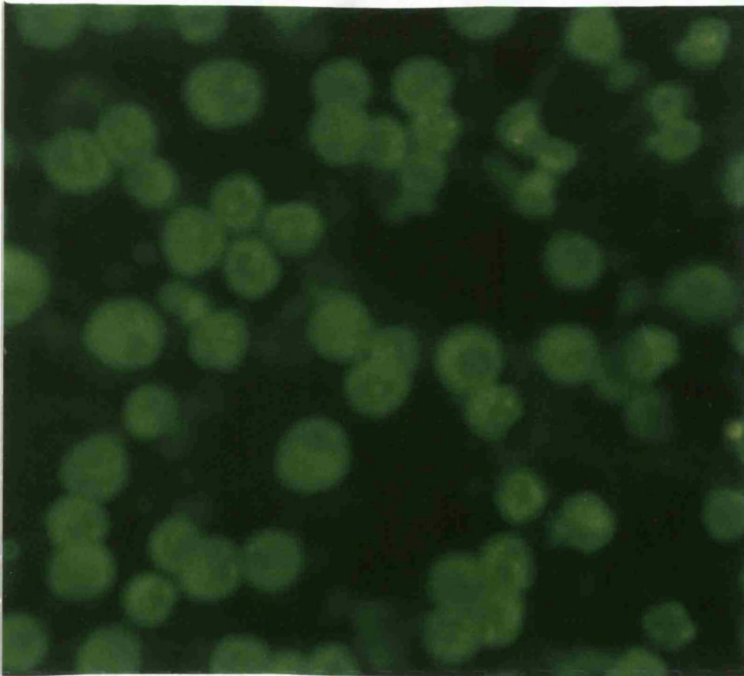


Figure 6.3 An example of the detection of anti-gB antibodies using immunofluorescence by end point dilution of the sera from a liver transplant recipient with primary HCMV infection shown in a). The negative control of cells infected with wild type baculovirus is shown in b).

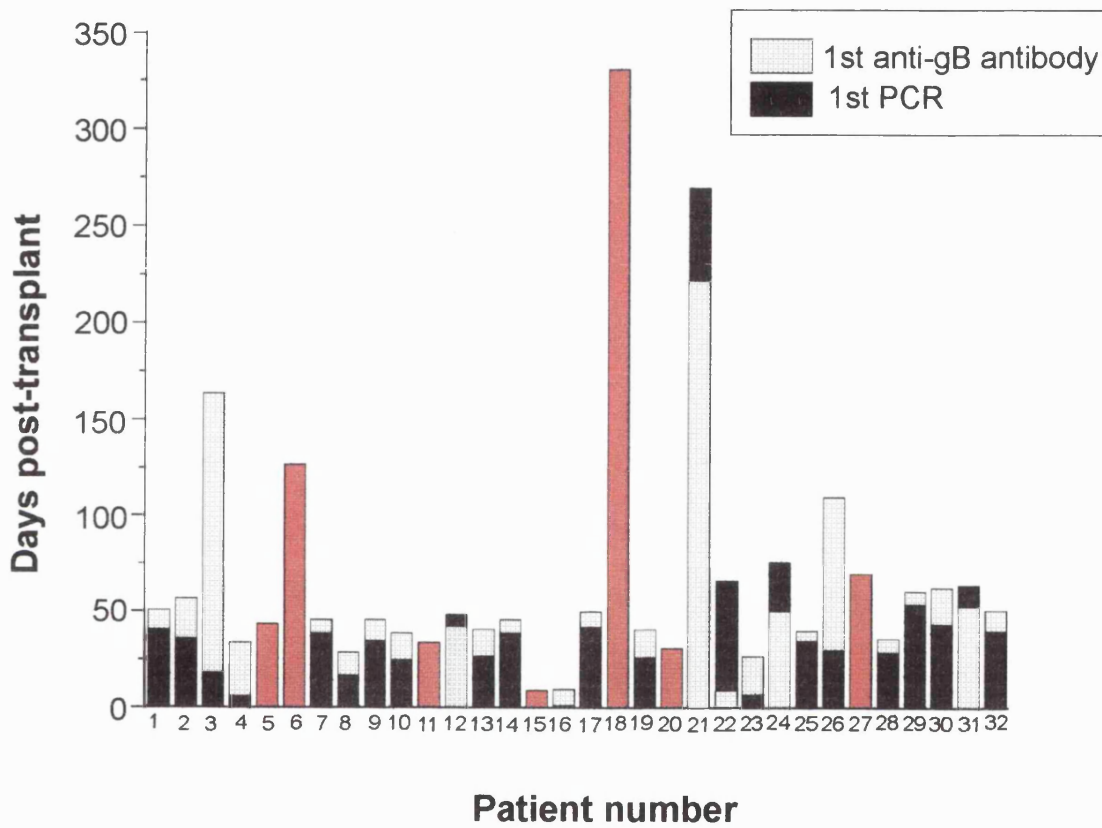


Figure 6.4 Relationship between the time to the first detection of HCMV DNAemia and the first detection of a four-fold rise in anti-gB antibodies post-transplant in 32 liver transplant recipients. The red bars indicate simultaneous detection of the first detection of HCMV DNAemia and a four-fold rise in anti-gB antibodies.

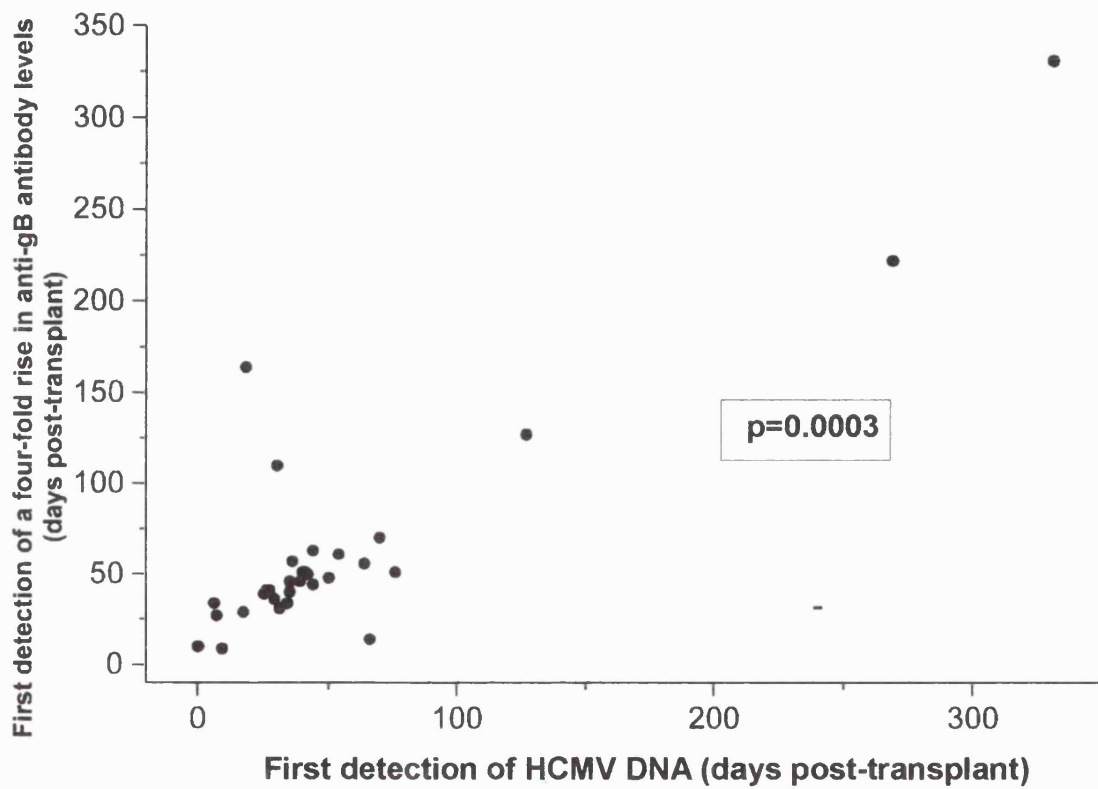


Figure 6.5 Relationship between the first detection of HCMV DNAemia and the first detection of a four-fold rise in anti-gB antibodies after liver transplantation. The p-value corresponds to Spearman's rank correlation coefficient.

6.3.3.1 Relationship between the time from the first detection of HCMV DNAemia to the detection of an initial rise in anti-gB antibodies

The data was examined further such that the time of the first appearance of HCMV DNAemia was taken as time 0. This analysis was performed in the context of HCMV disease, donor/recipient serostatus and administration of antiviral therapy. Figure 6.6 shows significant relationship between time from the first detection of HCMV DNAemia by PCR to a significant rise in anti-gB antibody level with respect to HCMV serostatus. This relationship was not observed in section 6.3 for the time to appearance of anti-gB antibodies and initial HCMV DNAemia post-transplant, indicating that the initial detection of anti-gB antibodies depends on the detection of HCMV DNAemia and recipient serostatus. The median time between the first episode of DNAemia to an initial rise in anti-gB antibody levels was 3.5 days (range, -52 - 80 days) in 18 HCMV seropositive recipients, which was significantly earlier than the median of 10 days (range, 0 - 146 days) for recipient seronegative individuals ($p < 0.05$ Mann - Whitney U test). These data indicate that patients with a primed immune system produce antibodies to gB following the first episode of DNAemia faster than patients that are naive to HCMV exposure. This relationship was independent of HCMV disease status and the initial HCMV viral load in blood, since recipient seropositive individuals had a significantly lower median viral load than recipient seronegative individuals (median= $10^{4.19}$ genomes/ml blood, range $10^{3.64}$ - $10^{7.52}$; median= $10^{5.34}$ genomes/ml blood, range $10^{3.59}$ - $10^{6.47}$; respectively: $p < 0.01$ Mann-Whitney U test).

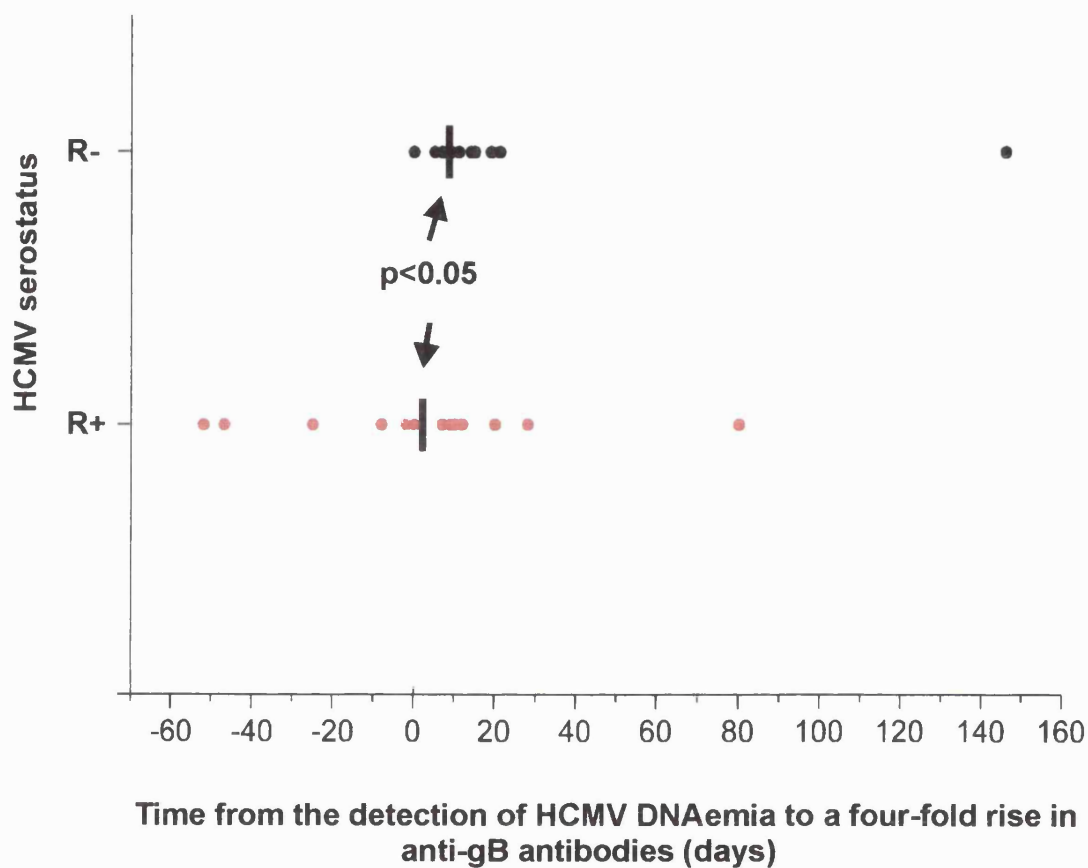


Figure 6.6 Relationship between HCMV serostatus, timing from the first detection of HCMV DNAemia to the first detection of a four-fold rise in anti-gB antibodies. The solid lines indicate medians for each group (Mann-Whitney U test). In this analysis, the detection of DNAemia was assigned as day 0.

6.3.4 Relationship between time to peak anti-gB antibody detection and time to peak HCMV viral load following liver transplantation

The timing of peak antibody levels and timing to peak HCMV viral load in blood was analysed in all 32 patients. The median peak viral load in the 32 patient cohort was $10^{4.98}$ genomes/ml blood (range $10^{3.59}$ - $10^{7.57}$), which was significantly higher in the 19 patients with HCMV disease (median= $10^{5.63}$ genomes/ml blood, range, $10^{3.66}$ - $10^{7.57}$) compared to the 13 asymptomatic patients (median= $10^{4.0}$ genomes/ml blood, range $10^{3.59}$ - $10^{4.93}$; $p < 0.01$ Mann-Whitney U test). Fourteen patients at risk of primary infection also had a higher median viral load (median= $10^{5.64}$ genomes/ml blood, range $10^{3.59}$ - $10^{7.57}$) than the remaining 18 HCMV seropositive recipients (median= $10^{4.40}$ genomes/ml blood, range $10^{3.64}$ - $10^{7.52}$; $p < 0.01$ Mann-Whitney U test). Peak viral load appeared at a median of 45.5 days (range 0 - 331 days) post-transplant, significantly earlier than a median of 59 days for peak anti-gB antibody levels (range 9 - 331; $p = 0.0156$, Mann-Whitney U test). Peak HCMV viral load preceded peak antibody levels in 23 patients, occurred at the same time in 5 patients and was detected later in 4 patients as shown in figure 6.7. Figure 6.8 shows the highly significant relationship between the time to peak HCMV viral load and time to peak anti-gB antibody response post-transplant ($R = 0.59$, $p = 0.0009$ Spearman's rank correlation). The peak HCMV viral load in blood also influences the level of anti-gB antibody response, since there was a highly significant correlation between the peak viral load and the level of peak anti-gB antibody levels for the 32 patients analysed ($R = 0.61$, $p = 0.0007$ Spearman's rank correlation) as shown in figure 6.9. However, the time to peak anti-gB antibody levels was unrelated to the peak viral load in blood and did not appear significantly earlier post-transplant in the 19 symptomatic patients compared to the 13 asymptomatic patients. Similarly, there was no significant association between HCMV serostatus, timing to peak anti-gB antibody levels or timing to peak HCMV DNAemia following transplantation.

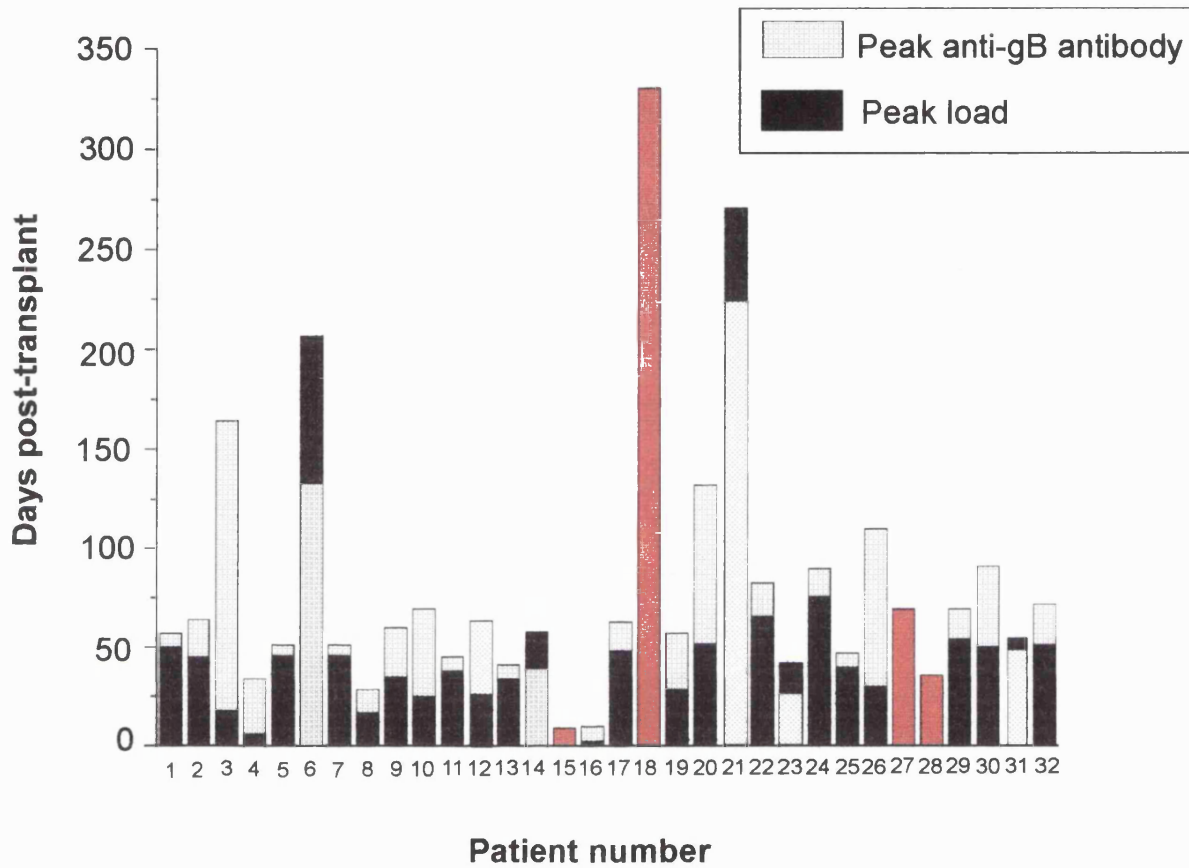


Figure 6.7 Time to the detection of maximum \log_{10} HCMV viral load in blood and peak anti-gB antibody levels in 32 liver transplant recipients. The red bars indicate simultaneous detection of peak viral load and peak anti-gB antibody levels.

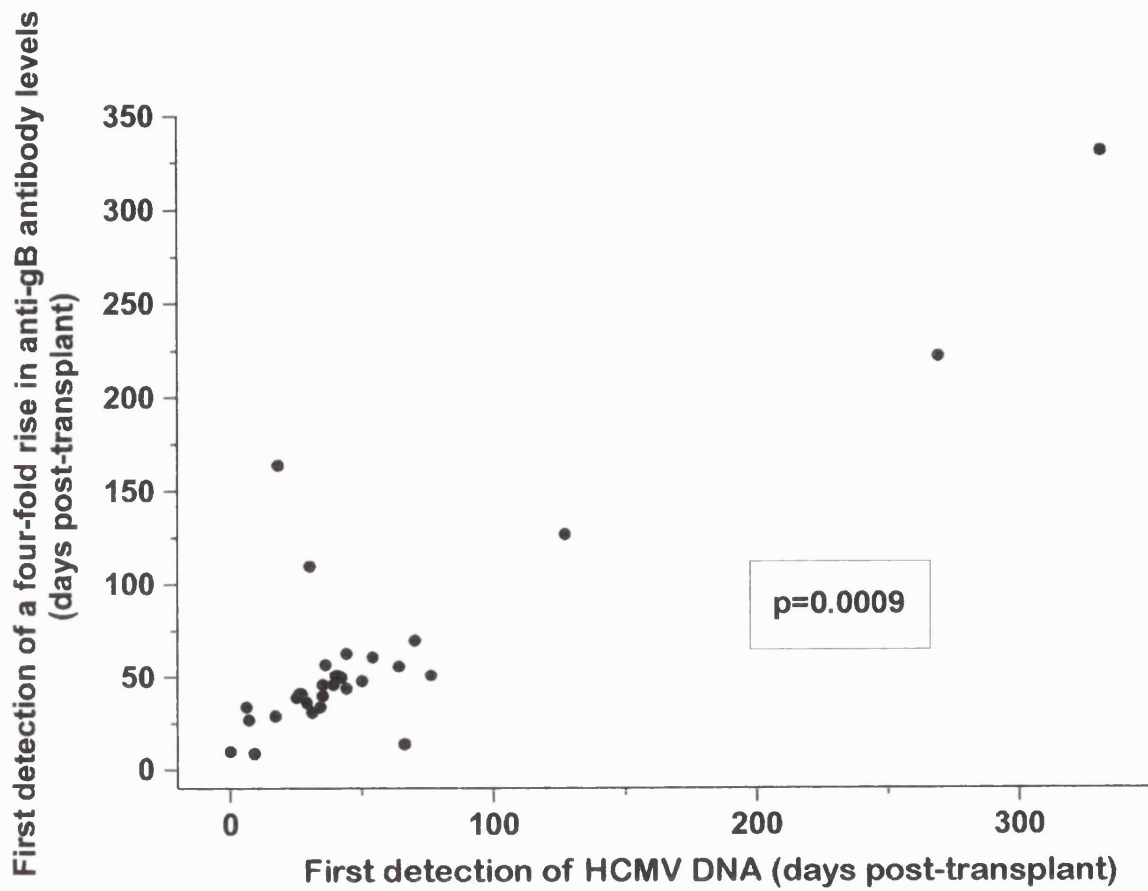


Figure 6.8 Relationship between the time to the detection of peak HCMV viral load and the detection of peak anti-gB antibody levels in 32 liver transplant recipients. The p-value corresponds to Spearman's rank correlation coefficient.

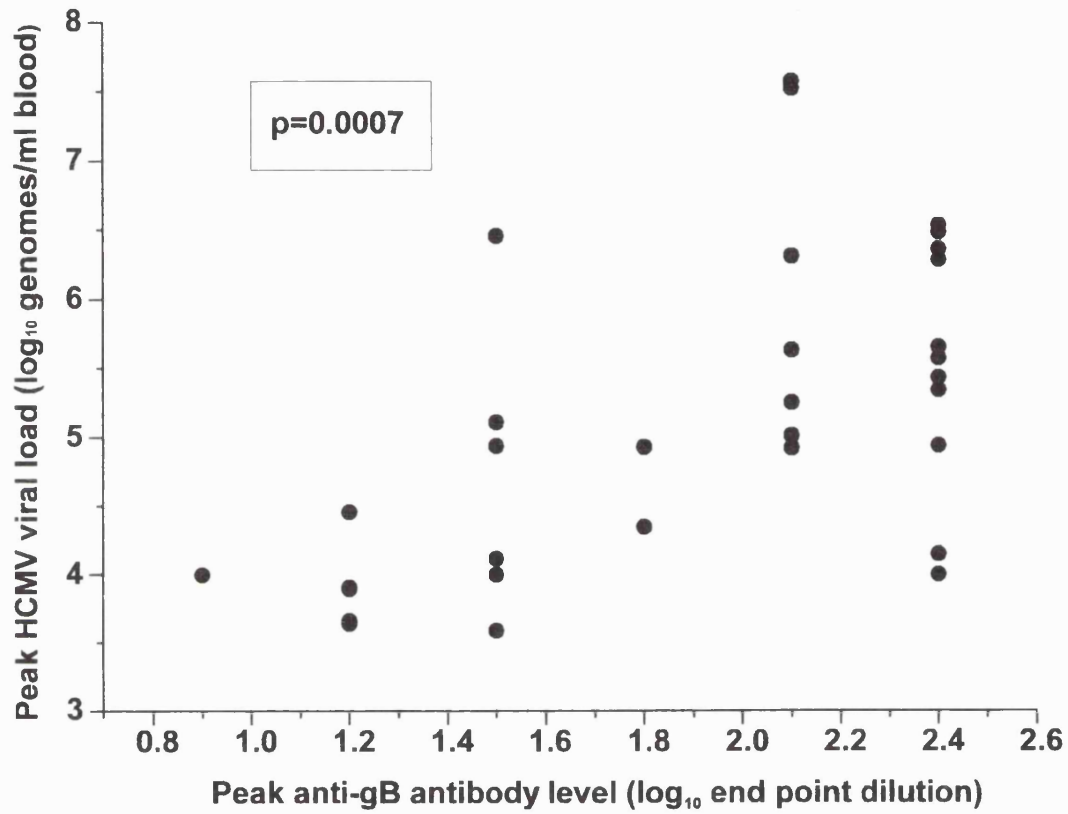


Figure 6.9 Relationship between peak HCMV viral load and peak anti-gB antibody levels in 32 liver transplant recipients. The p-value corresponds to Spearman's rank correlation coefficient.

6.3.4.1 Relationship between the time from the detection of peak HCMV viral load and peak anti-gB antibody levels

The timing from peak HCMV viral load to peak anti-gB antibody level was considered in the context of HCMV disease and donor/recipient serostatus, where peak viral load was taken as time 0. In recipient seropositive patients, peak anti-gB antibody levels were detected significantly earlier following the detection of peak viral load than in HCMV seronegative recipients (median=3.5 days, range -77 - 80; median=17.5 days, range 5 - 146; respectively: $p < 0.05$ Mann-Whitney U test) as shown in figure 6.10. Thus, as with the detection of the first episode of DNAemia (as described in chapter 2 section 2.9) and initial rise in anti-gB antibody levels, patients with a primed immune system were able to produce peak anti-gB antibody levels quicker than non-primed patients. This effect was independent of the HCMV disease status and HCMV viral load in blood also observed for the initial rise in antibody levels in response to the first episode of DNAemia detailed in section 6.3.3.

6.3.5 Relationship between peak HCMV viral load and timing to peak anti-gB antibody levels in patients receiving antiviral therapy

Of the 32 patients in this cohort, 14 recipient seronegative patients received antiviral therapy for HCMV disease 13 of whom received ganciclovir as monotherapy and one patient received both ganciclovir and foscarnet. The influence of antiviral therapy on timing to rise in anti-gB antibody levels following peak HCMV viral load was considered. The longitudinal profiles of HCMV viral load for the 14 patients are detailed in chapter 5 (figure 5.2). In all patients receiving antiviral therapy, the HCMV viral load was reduced considerably as discussed in chapter 5 section 5.3.4 (figure 5.5) of this thesis. Due to the timing of the treatment, only the influence of therapy on peak anti-gB antibody levels following peak HCMV viral load could be considered. Of the 14 patients receiving therapy, peak anti-gB antibody levels were reached prior to therapy in 2 patients, during therapy in 6 patients and occurred following therapy in 6 patients. The time difference from peak HCMV viral load to peak antibody in the latter two groups was analysed further. In 6 patients where peak anti-gB antibody levels were reached during treatment, the median time between peak HCMV viral load and peak anti-gB antibody levels was 7 days (range 5 - 17 days). This was significantly earlier compared to the 6 patients where peak anti-gB antibody levels were reached after antiviral therapy had stopped (median=23 days, range 7 - 80 days; $p < 0.05$ Mann - Whitney U test). The median viral loads between these two groups were not statistically different. This suggests that ganciclovir therapy does not have a pronounced effect on antibody levels.

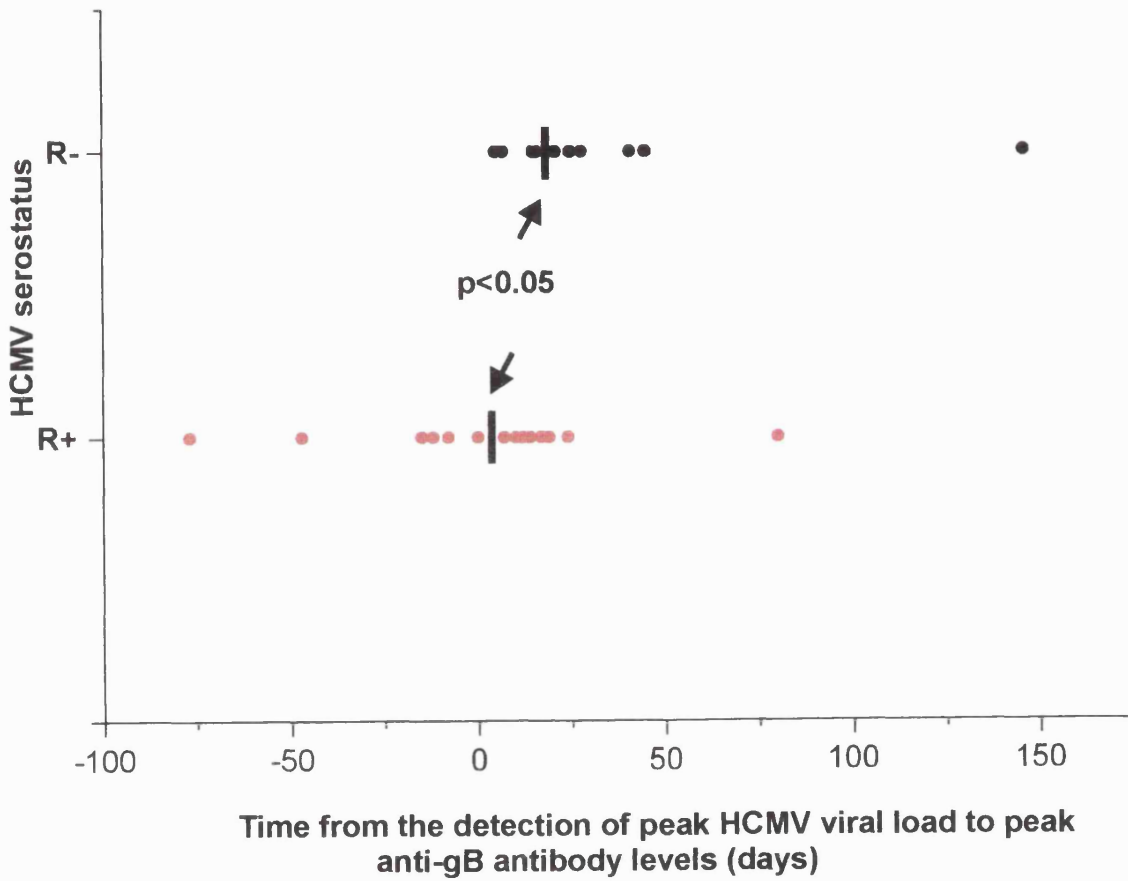


Figure 6.10 Relationship between HCMV serostatus and the time from peak viral load to peak anti-gB antibody levels. The solid lines indicate medians (Mann-Whitney U test). In this analysis, the detection of peak viral load was taken as day 0.

6.4 Discussion

The aim of this chapter was to investigate the dynamics of total HCMV IgG levels and anti-gB antibody levels in relation to the detection of HCMV DNAemia (as defined in chapter 2 section 2.9) and peak viral load following liver transplantation. Seventeen (94%) seropositive recipients had an initial IgG response prior to the detection of HCMV DNAemia compared to only 7% of the recipient seronegative patients. Similarly, peak total IgG was detected prior to peak viral load in 12 recipient seropositive patients, while 11 patients reached peak IgG levels following the detection of peak viral load in HCMV recipient seronegative patients. Thus, patients with a primed immune system were able to elicit an initial and a peak total HCMV IgG response faster than patients not previously exposed to HCMV. As previously reported in the literature, the total HCMV IgG response does not provide insight into the specific B-cell mediated immune response to HCMV infection (Marsano *et al*, 1990, Van der Geissen *et al*, 1990). In view of this, further analysis was performed to investigate the kinetics of specific anti-gB antibodies in relation to the detection of initial DNAemia and peak HCMV viral load following liver transplantation.

There was a trend for the detection of the first episode of HCMV DNAemia to appear prior to the detection of anti-gB antibodies following transplantation ($p=0.08$, Mann-Whitney U test). Unlike the detection of initial total HCMV IgG, there was a clear correlation between the time to the first episode of DNAemia and the detection of a significant rise in anti-gB antibodies following liver transplantation ($p=0.0003$, Spearman's rank correlation). The dynamics of the initial rise in anti-gB antibody levels was primarily dependent upon HCMV serostatus, such that patients with a primed immune system were able to elicit antibodies to gB more rapidly following the first episode of DNAemia than HCMV seronegative patients ($p<0.05$, Mann-Whitney U test). Patients undergoing a primary infection experienced a delay in the synthesis of antibodies to gB with a median of 11 days (range 0 - 146 days) following initial DNAemia. This relationship was independent of HCMV disease, and HCMV viral load in blood, where HCMV seropositive patients had a significantly lower initial viral load than recipient seronegative individuals.

There was also a highly significant correlation between the time to peak viral load and peak anti-gB antibody levels post-transplant. Indeed, peak HCMV viral load was detected significantly earlier than peak anti-gB antibody levels following transplantation ($p=0.016$, Mann-Whitney U test). Peak HCMV viral load was detected prior to peak anti-gB

antibody levels in 23 patients, at the same time in 4 patients and later in 5 patients. In addition the level of peak anti-gB antibodies was significantly associated with the level of peak HCMV viral load in blood. Indicating that the higher the viral load, the greater the level of anti-gB antibody response. Time dependent analysis showed this relationship to be independent of the occurrence of HCMV disease and the administration of antiviral therapy. However, as observed for the initial rise in antibody levels to gB in relation to first episode of DNAemia, this relationship was dependent upon the serostatus of the patients. Peak anti-gB antibody levels were detected significantly earlier following peak viral load in seropositive patients compared to the recipient seronegative group ($p < 0.05$, Mann-Whitney U test). As with the first detection of HCMV DNAemia, the timing from peak viral load to peak anti-gB antibody levels was not related to the peak viral load in blood, since seropositive patients had a significantly lower median viral load than patients undergoing a primary infection ($p < 0.01$, Mann-Whitney U test).

No significant difference was observed for the timing to peak anti-gB antibody levels following peak viral load for 14 patients receiving antiviral therapy for HCMV disease compared to 18 non-treated patients. However, peak anti-gB antibody levels were reached significantly earlier in patients where peak antibody levels to gB were detected during therapy than in patients where peak levels occurred after therapy had stopped ($p < 0.05$, Mann-Whitney U test). This data would suggest that antiviral therapy does not affect anti-gB body levels in response to DNAemia. As discussed in chapters 3 and 5 of this thesis, antiviral therapy has a profound effect on reduction of HCMV viral load. In conjunction with the reduction in HCMV viral load in the blood during therapy, peak anti-gB antibody levels were reached faster than in patients where therapy had stopped. Although the numbers of patients are small in this group, it is interesting to speculate that along with a reduction in viral load during therapy a recovery of the immune system allows a more rapid B-cell mediated immune response to follow. In contrast, in patients where therapy had stopped, HCMV viral load resumes causing a delay in anti-gB antibody response to viraemia.

These data are in agreement with Schoppel et al (1996) who also observed a delay in the synthesis of neutralising antibodies to glycoproteins in immunocompromised patients undergoing primary HCMV infection. The results from the study showed that the 6 liver transplant recipients at risk of either reactivation or reinfection did not experience a delay in the detection of antibodies to either non-neutralising or neutralising proteins. Four HCMV seropositive renal transplant recipients within the same study also synthesised

antibodies to neutralising glycoproteins concomitantly with the detection of HCMV viral DNA by PCR. In contrast, immunocompetent seropositive individuals had a qualitatively stable antibody profile for both neutralising and non-neutralising antibodies where antibody titres only fluctuated marginally over time. From this data it can be assumed that the kinetics of antibody synthesis to HCMV glycoproteins reflects viral replication or persistence of viral antigens in the lymphoid tissues in immunocompromised seropositive individuals. Thus, the delay in the detection of neutralising antibodies experienced by patients with primary HCMV infection is due to the lack of existing immunity including memory B-cells. It is also interesting to note that patients with primary infection have significantly higher HCMV viral loads, and as a result are more prone to the development of HCMV disease but do not elicit a more rapid B-cell mediated immune response. Thus, the mechanism of immune modulation in seropositive individuals with lower viral loads and fewer cases of HCMV disease may be different to that of patients with primary infection.

Several studies have highlighted the importance of the passive transfer of antibodies in limiting HCMV infection and disease in the transmission of the virus from mother to child during pregnancy (Stagno *et al*, 1982, Fowler *et al*, 1992, Adler *et al*, 1995, Boppana *et al*, 1996). The increased incidence and severity of congenital infection in children born to seronegative mothers with primary HCMV infection indicates that neutralising antibodies are unable to control virus replication. The delay in the synthesis of neutralising antibodies observed in organ transplant recipients with primary infection may account for viral dissemination and multiple organ involvement observed in these individuals. The increased viral dissemination could then lead to higher viral loads in blood and consequently an increased risk of HCMV disease. In contrast, in the recipient seropositive individual, antibodies which are synthesised concurrently with viraemia could reduce viral dissemination leading to lower viral loads and hence a reduced risk of HCMV disease. Antibodies to HCMV glycoproteins correlate significantly with neutralising antibody titres, the detection and level of these antibodies in relation to viral load levels may therefore act as a marker for viral dissemination. Indeed, the level of peak anti-gB antibody levels was significantly associated with the peak HCMV viral load in blood reflecting the level of HCMV DNA replication. Further analysis involving the relationship between the kinetics of neutralising antibody levels and viral load in seropositive and seronegative immunocompromised individuals are required to fully understand the mechanisms of immune protection via the B-cell arm of the immune system in these individuals. These studies may further aid the design of suitable sub-unit therapeutic

vaccines for the prevention of HCMV infection as well as providing valuable information into the pathogenesis of HCMV disease in the immunocompromised host.

Chapter 7.

Development of a Quantitative Competitive Reverse Transcriptase PCR assay (QCRT-PCR) for HCMV

7.1 Introduction

Traditional methods for the detection of HCMV have previously relied on the use of virus isolation and tissue culture techniques which, although reliable in their ability to detect infection, have the disadvantage of being both labourious and time consuming. The use of qualitative PCR assays for the detection of HCMV infection in a variety of patient populations has been established for some time. However, as reported by other authors, qualitative PCR can have the disadvantage of being able to detect latent infection in patients without apparent disease in the case of nested or extremely sensitive PCR assays. The sensitivity of qualitative PCR assays can be set to avoid this problem as well as providing prognostic information (Kidd *et al*, 1993). Alternatively, quantitative assays can differentiate between active and latent HCMV infections in immunocompromised patients as well as providing information into disease progression and antiviral efficacy (Fox *et al*, 1992 & 1995, Khun, *et al*, 1994, Drouet *et al*, 1995, Gerna *et al*, 1995). The discrimination between latency and active infection is paramount in immunocompromised patients to identify those most at risk of HCMV disease in order to prevent unnecessary exposure to toxic antiviral therapy. With respect to HCMV, quantitation of viral DNA is prognostic and provides insight into the pathogenesis of virus replication as discussed in chapters 3, 4, 5 and 6 of this thesis (Fox *et al*, 1992 & 1995, Bowen *et al*, 1995 & 1996, Mutimer *et al*, 1997, Gor *et al*, 1998).

With the advent of more sophisticated technology, techniques are being developed for the detection of messenger RNA to reflect ongoing viral replication using reverse transcriptase. Reverse transcriptase PCR (RT-PCR) has the advantage of identifying HCMV at the level of transcription, and many studies have described its use to identify the expression of an assortment of HCMV genes in a variety of clinical settings (Gozlan *et al*, 1992, 1993 & 1996, Bitsch *et al*, 1993b, Randhawa *et al*, 1994, Meyer-Konig *et al*, 1995, Von Laer *et al*, 1995, Boivin *et al*, 1996, Zhang *et al*, 1996, Nelson *et al*, 1996, Kondo *et al*, 1996, Movassagh *et al*, 1996, Wolff *et al*, 1996, Zhuravskaya *et al*, 1997, Gaeta *et al*, 1997). Three of these studies (Kondo *et al*, 1996, Movassagh *et al*, 1996, Zhuravskaya *et al*, 1997) have provided information into the latency of HCMV and the possible role of HCMV infection in myelosuppression experienced by bone marrow transplant recipients. Movassagh *et al* (1996) used RT-PCR for the detection of early and late mRNA in CD34⁺ and CD34⁻ cell lines isolated from cord blood to analyse the patterns of viral latency in progenitor cells. The results from this and a later study by Zhuravskaya *et al* (1997) both confirmed that bone marrow progenitor cells may act as a reservoir for latent HCMV with limited gene expression illustrating the utility of RT-PCR

for examining gene expression.

With respect to the clinical setting, Gozlan *et al* (1992) developed a RT-PCR assay for the detection of HCMV viral transcripts of pp65 and the major capsid protein (MCP). The lack of splicing events reported in this study of the pp65 gene, and its appearance at early times during infection in tissue culture experiments, led to the choice of MCP as the target gene for the RT-PCR assay. In a later study, Gozlan *et al* (1993) compared the use of RT-PCR for the detection of the late viral transcripts of the MCP gene with viral culture and DNA PCR from the peripheral blood of 102 HIV infected individuals. Although the sensitivity of the RT-PCR assay was lower compared to the other two techniques, the specificity of the assay was infinitely better at 94% compared to only 55% and 32% for culture and DNA PCR respectively (Gozlan *et al*, 1993). Gozlan *et al*, (1996) also reported the same specificity of the RT-PCR assay (94%) in 45 bone marrow transplant recipients compared to a specificity of 65%, 74%, and 84% for the detection of viral DNA by PCR, urine culture, and leukocyte culture respectively. Bitsch *et al* (1993b) and Randhawa *et al* (1994) both reported similar findings in smaller studies investigating the use of RT-PCR for the detection of viral transcripts in the peripheral blood leukocytes (PBL) of solid organ transplant recipients. The first study of eight HCMV infected renal transplant recipients examined the use of RT-PCR for the detection of the major immediate-early (MIE) and pp150 genes of HCMV. HCMV viral RNA was detected in the PBL of five patients with symptoms for the MIE gene and remained negative in three asymptomatic patients. Only one symptomatic patient was detected using the late structural protein pp150 as a target (Bitsch *et al*, 1993b). In the study by Randhawa *et al* (1994), of 21 solid organ transplant recipients, immediate early (IE) messenger RNA was detected in the peripheral blood of all seven symptomatic patients, the remaining 14 asymptomatic patients were negative for IE RNA irrespective of DNA detection indicating the potential prognostic value of this technique. In a more recent study, Boivin *et al* (1996) used RT-PCR for the detection of mRNA of glycoprotein H. Messenger RNA was detected in BAL samples from 11 patients with HCMV pneumonitis. In contrast, none of the 18 patients in this study that were shedding HCMV without HCMV disease had detectable mRNA in BAL samples (Boivin *et al*, 1996).

The choice of gene for the detection of HCMV viral transcripts is of great importance since immediate early transcripts may reflect latent virus or abortive replication, whereas the detection of late structural genes demonstrates a complete lytic cycle of viral replication. One study by Von Lear *et al* (1995) examined the detection of immediate early

and late pp150 HCMV viral transcripts in all the major leukocyte cell populations in the blood of renal transplant recipients with active HCMV infection. The results from this study demonstrated that all major leukocyte populations harboured early and late viral transcripts for HCMV including monocytes, PMNL (polymorphonuclear leukocytes) and PBMC's (peripheral blood mononuclear cells). The detection of early and late transcripts in these cell populations was indicative that replication and, indeed, infection with HCMV occurred in all cell types, but perhaps more poignantly illustrated the power of this technique to provide information into the pathogenesis of HCMV *in vivo*.

There have been several studies for the quantitation of HIV viral transcripts using RT-PCR methodology in attempt to elucidate further the dynamics of viral replication (Holodniy *et al*, 1991, Menzo *et al* , 1992, Scadden *et al* , 1992, Sei *et al* , 1994). All studies using quantitative RT-PCR assays for HIV have contributed valuable information into the pathogenesis and dynamics of HIV infection. However, there are very limited data using the same methodology for the quantitation of HCMV viral transcripts *in vivo* (Drouet *et al*, 1995). The following chapter describes the development and optimisation of a fully quantitative competitive RT-PCR assay (QCRT-PCR) using the late structural gene glycoprotein B (gB) as a target. In addition, this technique was applied in an *in vitro* experiment to compare the dynamics of both RNA transcription and DNA replication for the gB gene of HCMV in tissue culture.

7.2 Materials and Methods

The methodologies for the development of the quantitative competitive RT-PCR assay (QCRT-PCR) are described in detail in chapter 2 section 2.7. A brief description of the results obtained from the optimisation of the assay and the *in vitro* tissue culture experiment are described below

7.3 Results

7.3.1 Preparation of internal standards for QCRT-PCR assay

Initially, the 149 bp gB wild type and control DNA fragments were cloned into a pT3/T718U vector to produce sense RNA transcripts using T3 RNA polymerase by linearisation of the vector with Afl III as shown schematically in figure 7.1. Two separate cloning and transformation experiments were performed for the wild type and control 149bp gB fragments. A total of fourteen putative pT3/T718U gB clones were analysed for insertion of 149bp gB fragments by restriction enzyme digestion. The combined results of the double digest with EcoR1/HindIII and linearisation with Hpa1 of these two experiments is shown in figure 7.2. All 14 clones contained the 149bp gB fragment DNA (lanes 2 - 15, top panel), two of which were control DNA containing the mutated Hpa1 restriction site (lanes 14 and 15, bottom panel), while the remaining twelve clones (lanes 1-12) were wild type and not linearised with Hpa1. Two wild type and two control clones were subjected to DNA sequencing to establish the orientation of the inserted DNA. An example to show the mutated sequence of the Hpa1 restriction site for the control template is shown in figure 7.3. Having established that both inserts were inserted in the same orientation, the DNA was linearised with Afl III to allow the production of sense 573 nucleotide RNA transcripts with the enzyme T3 RNA polymerase (figure 7.1). To ensure complete linearisation of the vector templates prior to transcription, 2 μ l of the DNA templates were analysed on a 1% agarose gel shown in figure 7.4. The total RNA was treated with DNase 1 to remove the vector DNA template and the RNA purified.

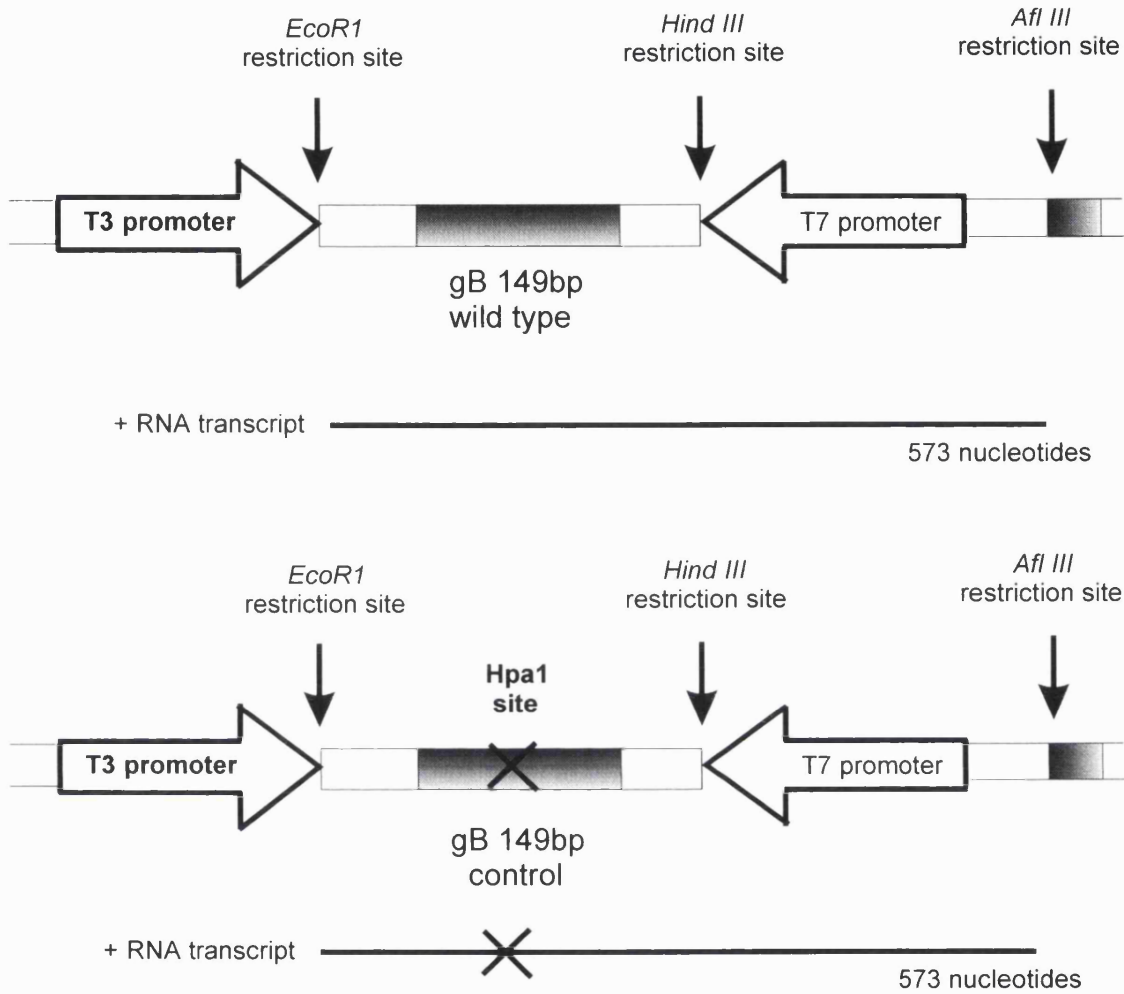
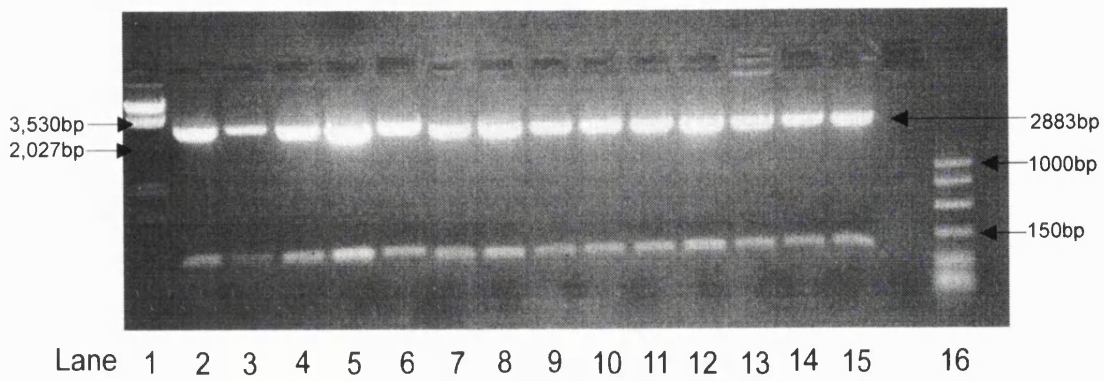


Figure 7.1 Schematic representation of the cloning of the 149bp gB fragments into the multiple cloning site of the pT3/T718U vector. The *EcoR1*, *Hind III* sites are shown, together with localisation of the RNA polymerase promoters (T3 & T7) and the *Afl III* restriction site for linearisation of the vector is also indicated.

Top panel

EcoRI/Hind III digest



Bottom panel

HpaI digest

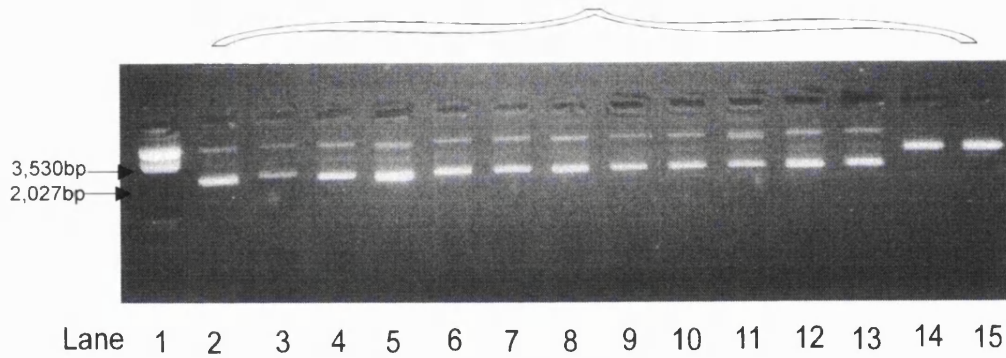


Figure 7.2 Restriction enzyme digestion of 14 putative pT3/T718U gB clones with *EcoRI* and *Hind III* (top panel) and *HpaI* (bottom panel). λ *Hind III/EcoRI* and PCR molecular size markers are also indicated.

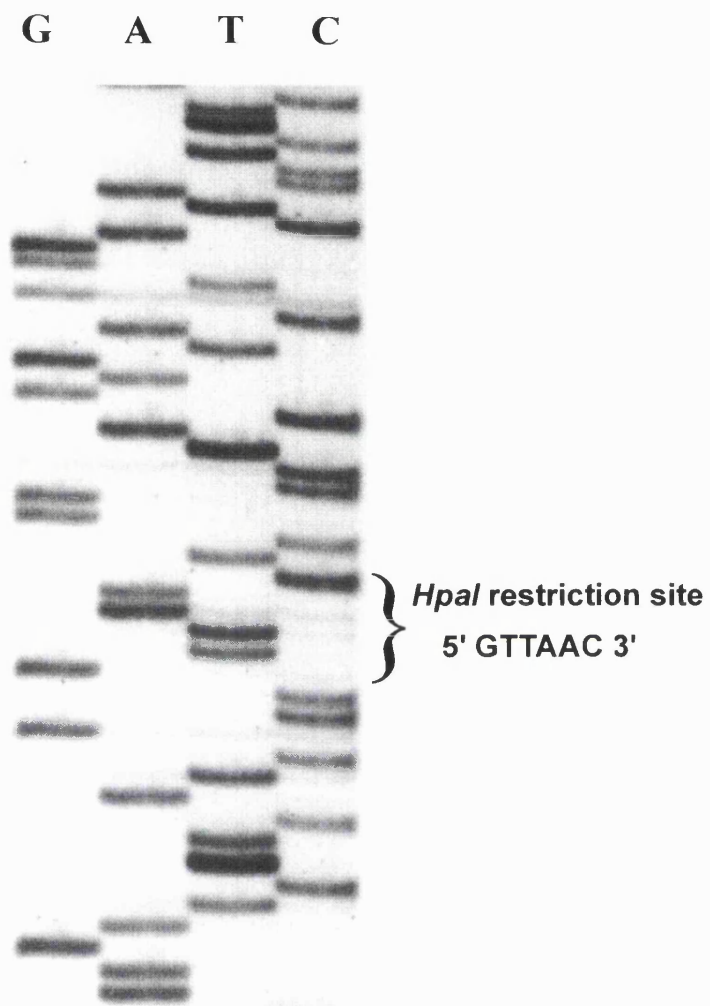


Figure 7.3 Example of an 8% Urea sequencing gel showing the localisation of the Hpa 1 restriction site present in the control pT3/T718U gB control clone.

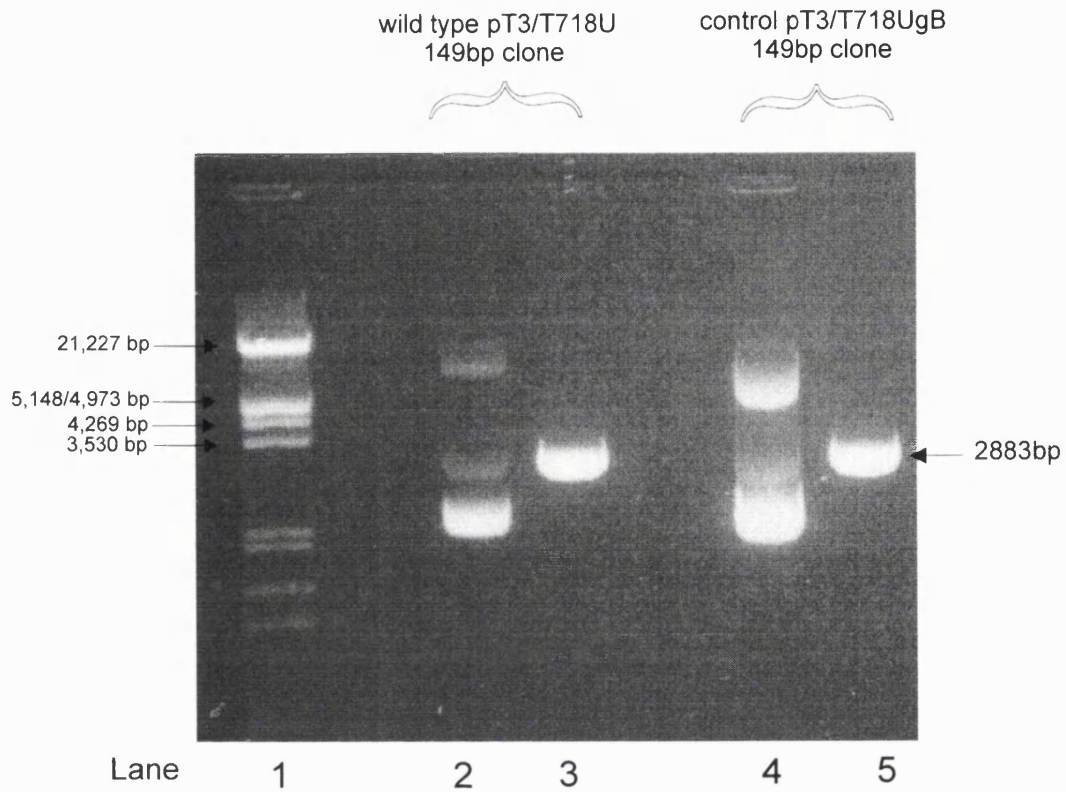


Figure 7.4 Linearisation of the recombinant wild type (lane 3) and control (lane 5) DNA templates with Afl III, the corresponding uncut plasmid vectors are shown in lanes 2 and 4. The molecular size marker λ Hind III/ EcoR1 is shown in lane 1.

2 μ l of each of the purified RNA templates was analysed on a 1.5% methyl mercury gel with an RNA marker to confirm the expected 573 nucleotide size of the wild type and control gB transcripts and to check for any degradation of the RNA during processing. Figure 7.5 shows that the purified wild type (lane 3) and control (lane 4) RNA transcripts were of the expected size and were not degraded during purification. Lane 2 shows the transcription reaction prior to purification and removal of the vector DNA template, the large molecular size band corresponds to the pT3/T718U vector (2883 bp).

7.3.2 Optimisation of the QCRT-PCR assay

The results of the DNA PCR of the diluted RNA templates within a range of between 10^8 - 10^3 copies are shown in figure 7.6, no contaminating DNA was detected within this range for either the wild type or the control RNA templates. Having obtained pure RNA, the QCRT-PCR assay was optimised for specific primer (gB1 & gB2) and magnesium sulphate concentration against 10^3 copies of the control RNA. The results of the primer titration are shown in figure 7.7, where the optimum concentration of the specific gB1 and gB2 primers was 10pmoles. Figure 7.8 shows the results from the magnesium sulphate titration using 10^3 copies of control RNA template with 10pmoles of both gB1 and gB2 primers, the optimum magnesium sulphate concentration was 1.75mM. At lower concentrations of magnesium sulphate there was a loss in sensitivity of the RT-PCR reaction (shown in lanes 6 - 10). The lower limit of detection of the QCRT-PCR assay was found to be 10^2 copies of control RNA template under optimised conditions using 10pmoles of each primer with 1.75mM magnesium sulphate, where one of the replicates had a band of the correct size (figure 7.9).

7.3.3 Determination of the dynamic range of QCRT-PCR assay

The optimum conditions of the QCRT-PCR assay described above were used to construct a calibration curve in order to determine the dynamic range of the QCRT-PCR assay. The control and wild type RNA templates were titrated against each other between 10^2 - 10^5 copies. The calibration curve is shown in figure 7.10, where the input RNA copy number was plotted against the calculated RNA copy number on a \log_{10} scale. The calibration curve shows a highly significant linear relationship over a broad dynamic range of between 10^2 - 10^5 copies of RNA ($Rho= 0.99$, $p=0.00005$; logistic regression analysis).

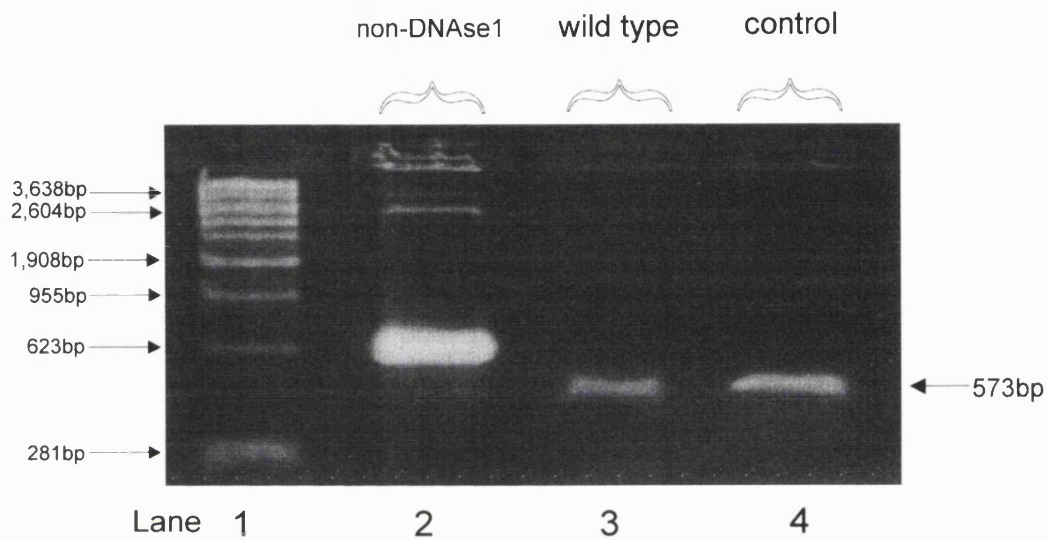


Figure 7.5 Methyl-mercury gel (1.5%) indicating the pure 573 nucleotide wild type and control RNA transcripts (lanes 3 & 4 respectively). Lane 2 shows 2 μ l of the control transcription reaction prior to DNase 1 treatment and RNA purification. Lane 1 shows the RNA molecular size marker.

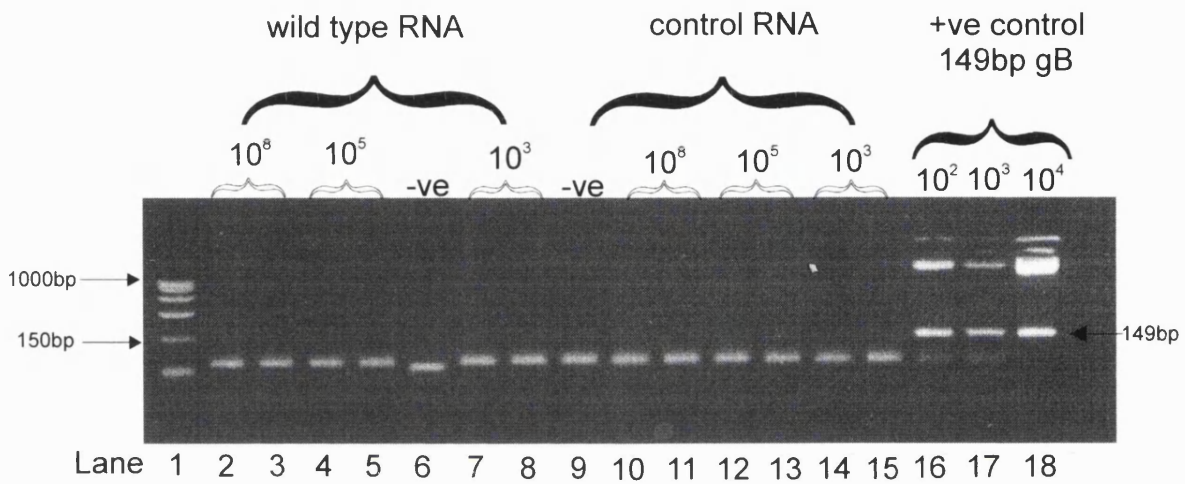


Figure 7.6 HCMV DNA PCR amplification of the control and wild type RNA transcripts ranging from 10^8 - 10^3 copies. Molecular size markers are also indicated (lane 1). Positive control gB 149bp is indicated in lanes 16 - 18. The larger molecular weight bands in the control lanes (16 - 18) are from the plasmid pUC18.

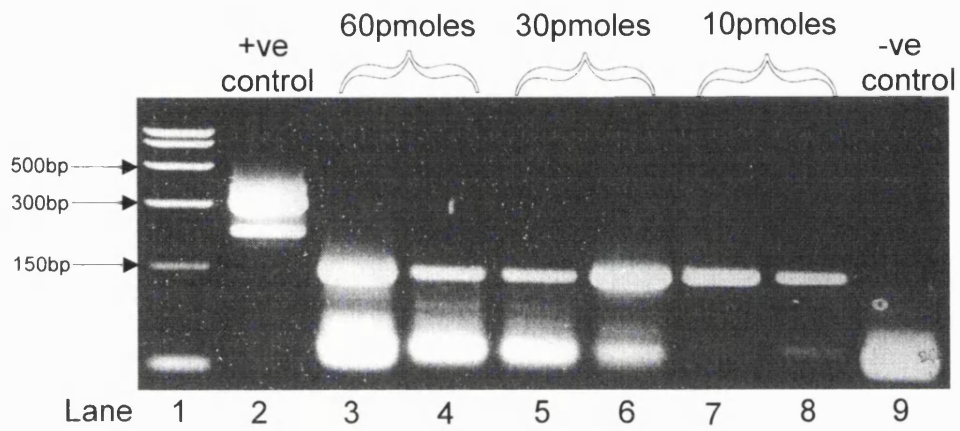


Figure 7.7 Primer titration of the specific primers gB1 and gB2 between 60 and 10 pmoles using 10^3 copies of the control RNA as template. Molecular size markers are also indicated (lane1).

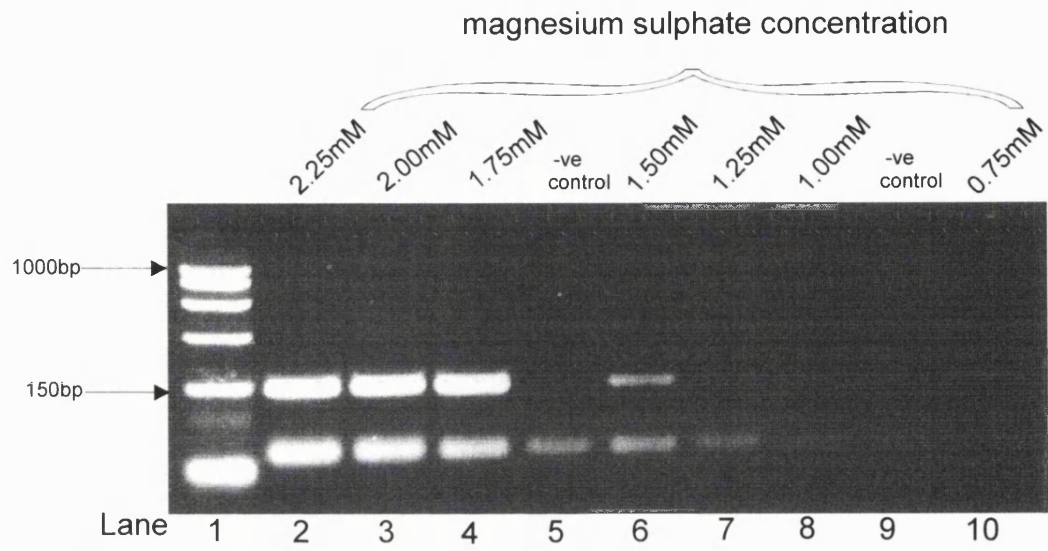


Figure 7.8 Magnesium sulphate titration between 2.25 and 0.75mM using 10^3 copies of the control RNA as template. Molecular size markers are also indicated (lane1).

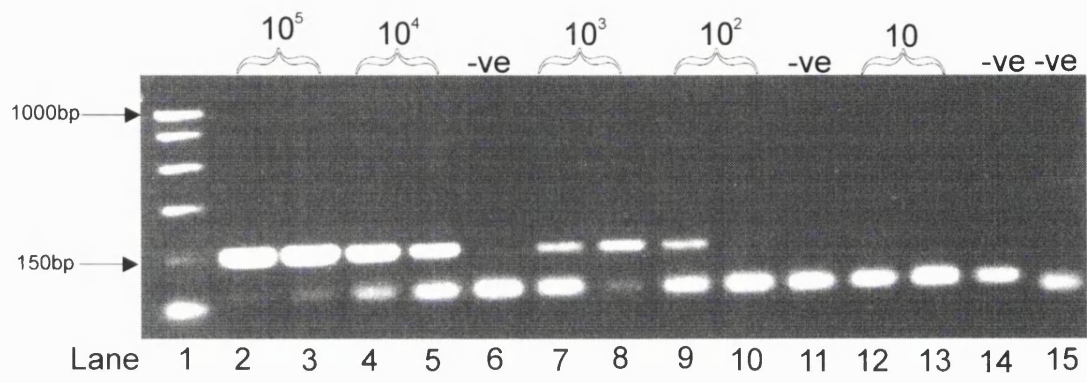


Figure 7.9 Control RNA template titration between 10⁵ and 10 copies of RNA template. Molecular size markers are also indicated (lane1).

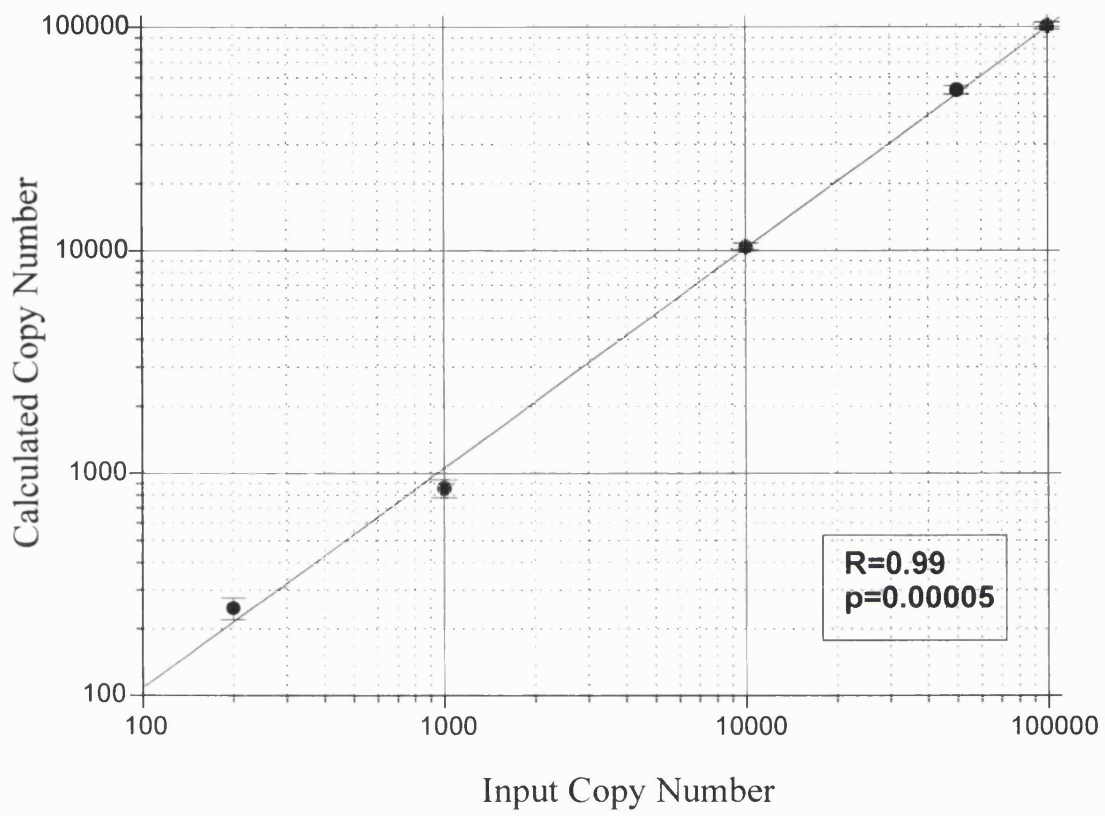


Figure 7.10 QCRT-PCR calibration curve.

7.3.4 Time course of gB DNA levels in HEL cells infected with the HCMV laboratory strain AD169

The levels of HCMV gB DNA were quantified using the QC-PCR assay and plotted against time for each different moi as shown in figure 7.11. The results indicate that in fibroblast cells, the levels of gB DNA increase exponentially with time up to the last time point of 96 hours post-infection with each moi. There was an initial drop in the quantity of HCMV gB DNA observed at 6 hours pi for each moi, which presumably reflects a lag phase for virus entry into the cells prior to the onset of DNA replication. The levels of HCMV gB DNA followed similar profiles at an moi of 0.1 and 0.5, at an moi of 0.01 there appeared to be less genomic DNA detected. Despite this observation, the levels of gB DNA followed comparable kinetics at each moi. When calculating the area under the curve, there was a progressive increase in the number of copies of HCMV gB DNA per hour with increasing virus inoculum, suggesting that HCMV DNA replication was proportional to the input viral inoculum as detailed in table 7.1. In addition, the doubling time of AD169 was calculated over a 48 hour period from 24 to 72 hours post-infection. The mean doubling time was 10.2 hours where doubling times of 11.94, 9.69 and 8.98 were calculated for moi's of 0.01, 0.1 and 0.5 respectively.

7.3.5 Time course of gB mRNA expression *in vitro* with laboratory strain AD169

The quantity of gB mRNA for each moi of AD169 was plotted against time post infection as shown in figure 7.12. The dynamics of gB mRNA expression followed similar kinetics for each moi. Irrespective of the moi, maximum gB mRNA was detected at 24 hours post infection (mean= $10^{6.08}$ copies/ μ g RNA; range, $10^{5.48}$ - $10^{6.83}$ copies/ μ g RNA) which dropped rapidly to stabilise at 72 hours post infection and remained constant at 96 hours. Surprisingly, mRNA was detected at 0 hours post infection, which may be due to contaminating DNA although qualitative DNA PCR analysis of the extracted RNA samples taken at 0 hours were negative. Lower levels of gB mRNA were detected at the lowest moi of 0.01 for each time point, the highest level being detected at an moi of 0.5. Details of the calculated area under the curve for gB mRNA are given in table 7.1. As for the calculated number of copies of gB DNA per hour, the number of copies of gB mRNA increased with the quantity of viral inoculum. This would suggest a dose dependent relationship for the level of HCMV replication.

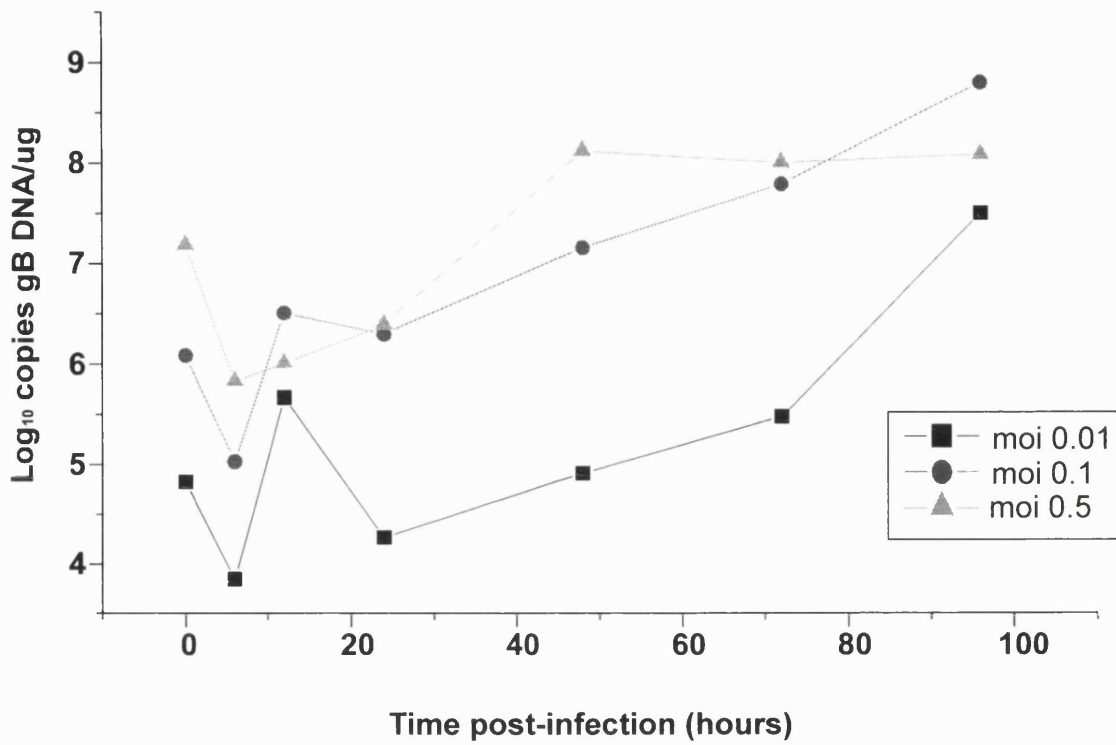


Figure 7.11 Levels of HCMV gB DNA produced during an *in vitro* time course of HEL cells infected with AD169

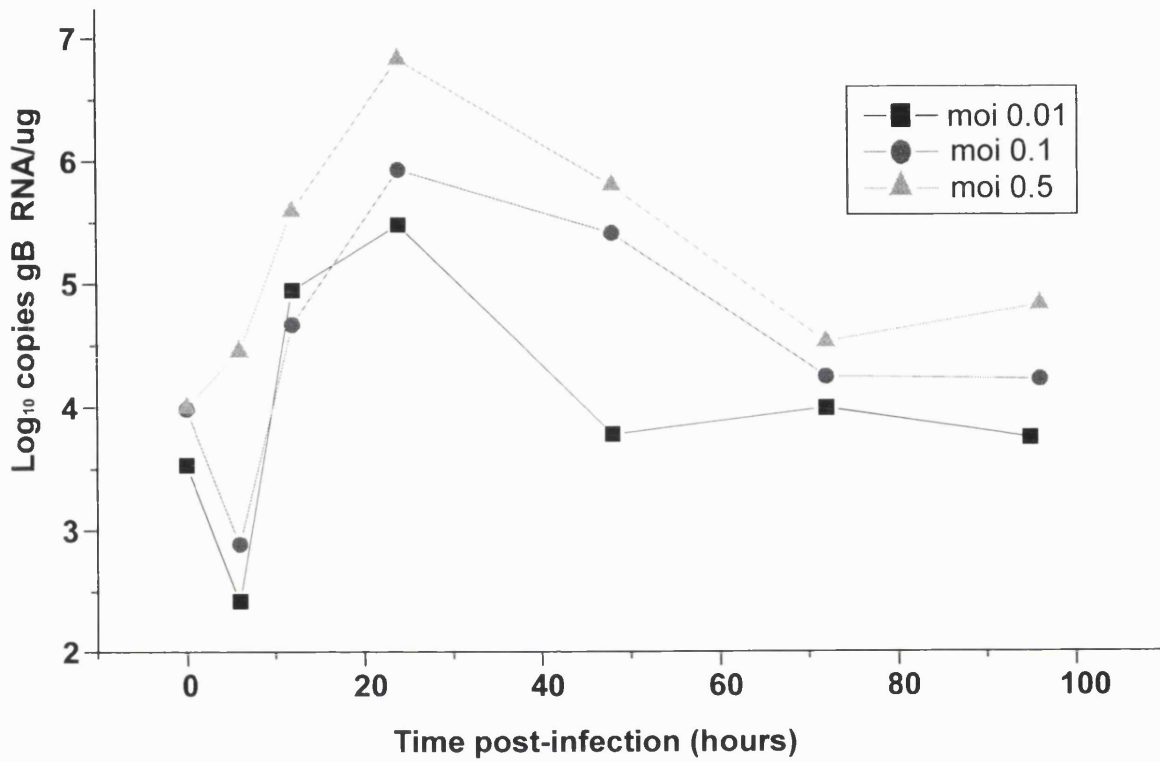


Figure 7.12 HCMV gB mRNA levels produced during an *in vitro* time course of HEL cells infected with AD169.

Table 7.1 Total area under the curve (AUC) for gB DNA and mRNA levels calculated from *in vitro* time course with AD169. Total AUC refers to the total HCMV viral load (\log_{10} genomes) calculated for 96 hours. The last column for each table shows the number of copies of either DNA or gB mRNA per hour.

DNA Quantitation:

MOI	Total HCMV viral load (\log_{10} copies DNA/ μ g/96 hrs)	Detection of peak gB DNA levels (hours)	\log_{10} DNA copies/hr
0.01	504.84	96	5.25
0.1	684.84	96	7.13
0.5	709.50	48	7.39

RNA Quantitation:

MOI	Total viral load (\log_{10} copies gBmRNA/ μ g/96 hrs)	Detection of peak gB mRNA(hours)	\log_{10} RNA copies/hr
0.01	399.66	24	4.16
0.1	460.32	24	4.80
0.5	517.71	24	5.39

7.4 Discussion

The aim of this chapter was to develop a fully quantitative reverse transcriptase PCR assay for the detection of HCMV mRNA. The late gene glycoprotein gB was chosen as a target for the assay in an attempt to discriminate between active virus replication and abortive or latent HCMV infection. One problem that may arise with the choice of such an unspliced late gene is the ability to differentiate between the viral transcript and contaminating genomic DNA (Gozlan *et al*, 1993). However, as detailed in the results section of this chapter, DNase1 treatment of the total RNA was sufficient to eliminate this potential problem. Indeed, qualitative DNA PCR amplification of the template RNA transcripts following DNase1 treatment yielded negative results for both internal control RNA sequences. The sensitivity of the quantitative RT-PCR assay was 10^2 copies, comparable to the QC-PCR assay for HCMV gB DNA following optimisation of primer and magnesium sulphate concentrations. The QCRT-PCR assay was also reliable over a broad dynamic range between 10^2 and 10^6 copies of gB mRNA illustrated by the generation of the calibration curve.

QCRT-PCR analysis was performed on total RNA extracted from a time-course of AD169 (HCMV wild type) infected human fibroblast cells at different multiplicities of infection, and was compared with the corresponding levels of HCMV DNA. The levels of genomic DNA and gB mRNA followed different kinetics, where DNA levels rose exponentially for each moi up to the last time point of 96 hours post infection. In contrast, the peak level of gB mRNA was reached at 24 hours post infection, falling at 48 hours and was sustained at a lower level 96 hours post infection for each moi. The levels of DNA and gB mRNA were dose dependent to the viral inoculum, with increasing levels of DNA and mRNA detected at increasing multiplicities of infection. The generation times calculated from the DNA quantitation decreased relative to the viral inoculum, where the mean generation time was 10.2 hours. This presumably reflects an analogous situation to that *in vivo*, however, further experiments examining cell types other than fibroblasts are required to fully elucidate the true kinetics of HCMV gB expression. Indeed, a recent report by Smuda *et al* (1998) showed that transcripts of different lengths with the UL54/55 gB/POL block (encoding glycoprotein B gene and the viral DNA polymerase genes of HCMV respectively) were transcribed at early and late times post-infection. The 8kb transcript was expressed at early times in the infectious cycle (12-24 hours pi), whereas the shorter 3.7kb transcript was detected at later times post-infection. The differential expression of these transcripts during the infectious cycle of the virus may explain the detection of peak gB mRNA levels as early as 24 hours post-infection observed here, and presumably

represents the expression of the 8kb transcript. In contrast, at later times, transcription of the 3.7kb transcript occurs which would account for the continued detection of gB mRNA expression at the later time of 96 hours pi.

To date, there are several reports using qualitative RT-PCR for the detection of both early and late transcripts of HCMV in the clinical setting (Bitsch *et al*, 1993, Gozlan *et al*, 1993, Randhawa *et al*, 1994, Meyer- Khonig *et al*, 1995, Zhang *et al*, 1995, Von Lear *et al*, 1995, Kondo *et al*, 1996, Nelson *et al*, 1996, Wolff *et al*, 1996). These studies highlight the application of such a technique for the detection of active HCMV replication, however, as with qualitative detection of HCMV DNA, the reliability to provide prognostic information is questionable. Despite this, the advantage of RT-PCR for the detection of late transcripts allows the distinction between latent and active virus replication unlike the use of qualitative DNA PCR (Gozlan *et al* 1993, Bitsch *et al* 1993b, Meyer-Konig *et al* 1995, Von Lear *et al* 1995, Nelson *et al* 1996). Indeed, the versatility of this technique has recently provided important information into the pathogenesis of HCMV latency (Kurz *et al*, 1997, Kondo *et al*, 1996). Kondo *et al* (1996) utilised RT-PCR to identify a novel latency associated transcript in bone marrow-derived myeloid progenitor cells derived from bone marrow aspirates of healthy seropositive donors and naturally infected individuals suggesting an important site of HCMV latency. While the latter of these two studies identified the lungs in MCMV infection as a major site of latency and recurrence following infection.

Initially, the QCRT-PCR assay was used to analyse the temporal expression of HCMV gB *in vitro* to assess suitability and reliability of the assay in the clinical setting. The application of such a technique over qualitative detection of viral RNA are numerous. Indeed, longitudinal quantitative analysis of gB mRNA in serial blood or tissue samples and possibly different cell types from immunocompromised patients will allow the pathogenesis of virus replication to be further elucidated. In addition, as with quantitation of HCMV DNA discussed in chapters 3, 4, 5, and 6 of this thesis, this will not only help to define threshold levels upon which to direct treatment regimens, but will also provide invaluable information into the effects of antiviral therapy upon virus replication. Indeed, during treatment with antiviral therapy, HCMV DNA was still detected in the blood of liver transplant recipients, albeit at lower levels as described in chapter 5 of this thesis. In contrast, upon treatment with antiviral therapy RNA levels in the blood are reduced to undetectable levels reflecting the dramatic effect of therapy on virus replication (Gaeta *et al*, 1997). Thus, the use of a QCRT-PCR assay may be more informative as a measure

of antiviral efficacy in the immunocompromised host.

Chapter 8.

General Discussion

The aim of this thesis was to investigate the pathogenesis of human cytomegalovirus (HCMV) disease in the immunocompromised host and to elucidate the most important factors that can influence clinical outcome by the identification and quantification of risk factors for HCMV disease. The frequency by which HCMV infection leads to disease is elevated in patients whose immune system is impaired either as a result of immunosuppressive therapy in the case of transplant recipients, an immature immune system as for congenitally infected infants, or finally as a result of immune dysfunction in AIDS patients. Primary infection in the immunocompetent host is usually followed by latency, however, in the immunocompromised host, reactivation of latent strains due to immunosuppression can also result in HCMV disease. To date, few studies have used a fully quantitative competitive PCR assay to measure viral load in a variety of patient populations in order to provide prognostic information. This thesis examined the modulations in HCMV viral load in immunocompromised individuals in the context of other risk factors for disease in an attempt provide prognostic and pathogenic information and where applicable to assess the effects of antiviral therapy on HCMV virus replication.

HCMV infection remains the major source of congenital viral infection with an incidence of approximately 0.2 - 2% of all live births in the USA. Establishment of HCMV disease can occur some years later in the infant, manifest in the severest form as sensorineural hearing loss and or mental retardation. Diagnosis at birth relies on virus isolation within the first three weeks of life and is not indicative of the severity of clinical outcome. The difficulty at present in defining congenital infection is not only identifying infants at risk, but also the long term follow up required to assess virological markers for disease and to assess antiviral efficacy. More recent studies have focused on alternative PCR based methods for prenatal diagnosis of congenital HCMV infection by analysis of amniotic fluid (Donner *et al*, 1993, Hogge *et al*, 1993, Revello *et al*, 1995, Lipitz *et al*, 1997). The first pioneering study to observe a significant association between increased HCMV viral titre in urine of congenitally infected infants and poor clinical outcome was observed 23 years ago by Stagno *et al* (1975). Since then there have been few subsequent studies to examine this relationship using modern molecular biological techniques. In chapter 3 of this thesis the viral load fluctuations in the urine of congenitally infected infants receiving antiviral therapy was compared with non-treated infants. The antiviral effect on HCMV shedding in the urine was dramatic - 66% of the infants receiving ganciclovir became PCR negative during treatment. In infants receiving ganciclovir, the pre-treatment viral load was significantly lower than the post- treatment viral load when considering all samples analysed despite a median of 170 days after therapy had stopped. This

illustrates further the sustained antiviral effect of ganciclovir therapy on virus replication. In contrast, the viral load in the non-treated infants did not fall below $10^{4.0}$ genomes/ml urine and infants experienced continued viral shedding during surveillance. Thus, for a relatively short period of ganciclovir therapy a profound long term effect on viral load suppression in urine was observed. This may have important implications when considering new treatment regimens with ganciclovir for symptomatic congenitally infected infants to reduce the severity and incidence of ensuing sequelae. The estimated mean viral clearance for infants receiving ganciclovir was rapid at 1.65 days and was independent of the pre-treatment viral load. The rapid clearance of virus suggests that much of the virus detected is a consequence of newly infected cells. The latter process is interrupted by ganciclovir which will act to reduce the number of virus particles produced to initiate new infection. Thus, the clearance of virus presumably reflects immune mediated (specific and non-specific) mechanisms. It will be interesting to determine the nature of these mechanisms and to compare them quantitatively with those present in transplant recipients following ganciclovir intervention (see later).

To date, the results of one phase II study for the treatment of symptomatic congenital HCMV infection with ganciclovir have shown a sustained reduction in viral shedding in urine (Whitley *et al*, 1997). A more favourable clinical outcome was observed in 16% of babies with improved hearing at six months follow up. These preliminary data are encouraging, however, prolonged follow up is required to assess the potential long term toxic effects of this therapy on the development of the child. It is important, therefore to identify infants at risk early on so that they can be targeted for antiviral intervention with ganciclovir, thus minimising exposure to this very toxic agent. The ability to diagnose congenital infection prenatally by PCR analysis of amniotic fluid may prove advantageous in future studies but care must be taken to ensure consistency in the timing of sampling (Donner *et al*, 1993, Hogge *et al*, 1993, Revello *et al*, 1995, Lipitz *et al*, 1997). In order to establish the value of QC-PCR as a prognostic marker for the development of severe sequelae will also require analysis with prolonged follow up of larger groups of congenitally infected infants. Nevertheless, it is easy to see the potential of this rapid non-invasive technique over conventional methods to provide valuable quantitative information in the context of congenital infection and antiviral efficacy. These data, together with identification of other risk factors for disease, including the immune status of the mother, time to acquisition of the virus either in utero or perinatally by prenatal detection of HCMV may prove to be of significant prognostic value in future studies.

In chapter 4 of this thesis, the use of quantitative PCR to monitor HCMV load in the urine of renal transplant recipients in the context of other risk factor for HCMV disease was examined. Renal transplant recipients are at risk of HCMV disease within the first three months following transplantation as a result of immunosuppression. HCMV infection results by acquisition of the virus from the donor organ manifest as primary infection, reactivation of endogenous latent HCMV or finally by reinfection of donor strains in pre-immune individuals. Due to the short supply of organs it is not always possible to avoid primary infection by transplantation of a seronegative donor into a seronegative recipient. Therefore, it is important to identify patients most at risk of disease in order to administer the appropriate antiviral intervention. In the prospective study presented in chapter 4 and in other studies, the mere presence of HCMV in the urine is of little value when assessing clinical outcome of the patients (Kidd *et al*, 1993). However, elevated viral load was significantly associated with HCMV disease when compared to asymptomatic patients ($p < 0.01$). The difference between the maximum median viral loads in symptomatic versus asymptomatic patients corresponded to 2.44 \log_{10} genomes/ml urine. More importantly, the data illustrated that the quantity as opposed to detection of the virus was crucial to provide prognostic information (Fox *et al*, 1995, Cope *et al*, 1997a). Furthermore, in the multivariate model, elevated viral load remained the single most important independent risk factor for HCMV disease ($p = 0.04$).

The identification of other risk factors for HCMV disease in the renal transplant recipients described in chapter 4, including the presence of HCMV viraemia and receipt of an organ from a HCMV seropositive donor, are in agreement with previous studies (Fox *et al*, 1995, Iragori *et al*, 1993, Schafer *et al*, 1993, Khun *et al*, 1994). The risk factor of donor seropositivity was negated after controlling for HCMV viral load suggesting that this is the mechanism through which this risk factor operates. Similarly, the protective effect of recipient seropositivity was negated after controlling for viral load in the multivariate analysis. In other words, patients with primary infection have disease due to higher viral loads, whilst recipient seropositive patients are protected because they have a low viral load. Despite the identification of elevated viral load in urine as an independent risk factor for disease, there was also a possible association with the presence of HCMV viraemia ($p = 0.07$). Fifteen (43%) of the renal transplant patients were viraemic 73.3% of whom developed disease. These data show that not all renal transplant recipients with HCMV disease have viraemia, but in those that do, there is a significantly greater risk of developing HCMV disease. Thus, the value of continued monitoring of patients by QC-PCR in urine to identify patients with elevated viral loads who are more likely to develop

disease is warranted.

The computed probability of disease curve for viraemic and non-viraemic renal transplant recipients showed that at critical viral loads relatively modest increases in viral load increased the probability of disease dramatically. Thus, an increase in viral load from $10^{5.0}$ genomes/ml urine to $10^{5.75}$ genomes/ml urine in viraemic patients resulted in an increase from 10% to 80% in the probability of HCMV disease. Previous studies have identified systemic detection of HCMV is associated poor prognosis (Schafer *et al*, 1993, Bitsch *et al*, 1993a, Khun *et al*, 1994, Fox *et al*, 1995). The data presented in chapter 4, unlike previous studies, provides detailed longitudinal HCMV viral load data in the context of the other risk factors for disease including the detection of HCMV viraemia and donor/recipient serostatus (Cope *et al*, 1997a). Perhaps the most interesting concept obtained from this data set is the possibility of directing the administration of antiviral therapy based on the establishment of HCMV viral load threshold levels. Indeed, this could lead solely to pre-emptive administration of antiviral therapy once a certain HCMV viral load threshold is reached. However, for this to become an established protocol, further studies involving larger numbers of renal transplant patients are required.

In chapter 5 of this thesis the importance of elevated viral load in the blood of liver transplant recipients was investigated. As shown for the urine renal transplant recipients, HCMV disease was associated with elevated HCMV viral load in blood in liver transplant recipients ($p < 0.0001$). However, the difference between medians corresponded to a $1.35 \log_{10}$ genomes/ml blood, significantly lower than that observed in the urine of renal transplant recipients ($p < 0.05$). This implies that the quantity of systemic HCMV viraemia is more significant for the onset of HCMV disease by seeding of virus to distant organs. Indeed, recent analysis of 31 renal transplant recipients with HCMV viraemia at our center revealed a similar difference of $1.58 \log_{10}$ genomes/ml blood between the maximum median viral load in blood of patients with and without HCMV disease (Hassan-Walker *et al*, manuscript in preparation). The disease probability curve generated from the univariate analysis of 47 liver transplant recipients revealed a similar situation to that observed for renal transplant patients. The risk of HCMV disease rose dramatically from 10% at $10^{4.75}$ genomes/ml of blood to 80% at a viral load of $10^{5.5}$ genomes/ml blood. As suggested for renal transplant recipients, the emphasis of antiviral pre-emptive therapy could be aimed at reducing HCMV viral load to manageable levels, a more realistic option than completely eliminating virus replication. However, the use of threshold levels to predict development of HCMV disease and direct antiviral therapy in the transplant setting

requires extensive further studies.

The complexity of HCMV disease pathogenesis in liver transplant recipients was demonstrated by the identification of other risk factors for HCMV disease including donor/recipient serostatus, and the administration of augmented immunosuppressive therapy. Donor serostatus as a risk factor for HCMV disease was negated after controlling for viral load in the multivariate analysis. Thus, it can be assumed that viral load is the mechanism through which this risk factor operates. The dominant effect of increased viral load on the development of HCMV disease was illustrated in the multivariate analysis, where increased viral load was identified as a significant independent risk factor for HCMV disease ($p=0.005$). The odds ratios were not significantly altered after controlling for either donor/recipient serostatus or the administration of augmented methylprednisolone in liver transplant patients. However, augmented immunosuppression with methylprednisolone was an independent risk factor for disease. The relevance of this observation will be discussed later.

Collectively, the results obtained from analysis of HCMV load in the urine of renal and the blood of liver transplant recipients in this thesis demonstrate that the detailed longitudinal analysis of HCMV viral load in the context of HCMV serostatus can provide important information into the pathogenesis of HCMV disease. For example, in patients at risk of either reactivation or reinfection (D+R+) an intermediate risk of disease was observed. The data revealed that patients with viral loads above the median of the D+R+ group were at greater risk of HCMV disease presumably experiencing a situation analogous to primary infection with exogenous virus from the donor. Previous studies have confirmed that infection with donor strains of HCMV during transplantation is associated with an increased risk of HCMV disease (Grundy et al, 1988). In contrast, D+R+ patients with viral loads below the median of the D+R+ group had a significantly lower risk of HCMV disease and it is presumed these patients were experiencing reactivation of endogenous HCMV. Indeed, none of the renal and only one of the liver transplant recipients who had viral loads below the median viral load in the D+R+ group experienced HCMV disease. However, to confirm this hypothesis detailed analysis by restriction fragment length polymorphism studies of larger groups of patients at risk of either reactivation or reinfection are required.

The utility of QC-PCR to identify increased virus load as a risk factor for HCMV disease has been clearly demonstrated from the results obtained in chapter 4 and 5 of this thesis

(Cope *et al*, 1997a & 1997b). The application of this method to measure the effect of antiviral therapy on virus load has also been shown for congenitally infected infants in chapter 3. In chapter 5, a similar sustained antiviral effect of ganciclovir therapy on HCMV load in the blood of liver transplant recipients, was observed, with 84.6% of patients becoming PCR negative during treatment and 57.2% of the liver patients remaining PCR negative after therapy had stopped. The mean reduction in HCMV viral load was 2.10 \log_{10} genomes/ml blood, which was comparable to mean reduction of 3.94 \log_{10} genomes/ml urine observed for congenitally infected infants receiving ganciclovir therapy. The mean clearance rate of the virus in the blood of liver transplant recipients was of 2.1 days. When the data was analysed statistically there was no significant difference between the clearance rates obtained for congenitally infected infants (mean=1.65 days) or liver transplant recipients. The pre-treatment viral load was not related to the $t_{1/2}$ values in either congenitally infected infants or in liver transplant recipients receiving ganciclovir therapy. These data suggest that the quantity of virus alone does not necessarily dictate the ability of liver transplant recipients to clear systemic HCMV infection. Indeed, there are other factors which could influence the ability of the host to clear virus when treated with antiviral therapy, including the source of the virus as either donor or host derived, and the immune status of the individual. Unfortunately, the numbers of patients available for analysis in chapter 5 were insufficient to analyse the influence of these parameters on virus clearance rates. Future studies could investigate the effect of HCMV serostatus and the administration of immunosuppressive therapy on the ability of the host to clear virus to test this hypothesis.

Previous studies in liver transplant recipients have identified an increase in the incidence of HCMV disease with treatment of rejection episodes with augmented immunosuppressive therapy such as ATG, OKT3 and methylprednisolone, although the pathogenic mechanisms remain to be fully elucidated (Stratta *et al*, 1991, Portela *et al*, 1995, Hadley *et al*, 1995, Lao *et al*, 1997). In chapter 5, the effect of augmented immunosuppressive therapy on the incidence of HCMV disease in liver transplant recipients was analysed as a risk factor for disease. Although, patients received triple therapy following transplantation, the main treatment for rejection was administration of augmented methylprednisolone. Statistical analysis revealed receipt of augmented methylprednisolone was an independent risk factor for disease after controlling for either donor seropositivity or increasing viral load. The effect of immunosuppressants such as OKT3 and ATG on the immune system is to specifically target the T-cell mediated immune response. Corticosteroids, such as methylprednisolone, have a global effect

reducing both circulating T-cell numbers and blocking the differentiation of monocytes to macrophages by initially suppressing the synthesis of IL-1 by monocytes. As a consequence, the anti-inflammatory effect of this corticosteroid is powerful at reducing rejection episodes in transplant recipients. However, NK cell numbers and activity are unaffected by methylprednisolone.

With an impaired immune system due to administration of immunosuppressive therapy the T-cell mediated immune response, which is predominately directed to pp65 of HCMV, is also diminished preventing the host from surveying and subsequently controlling virus replication. Interestingly, the disease probability curve generated in chapter 5 revealed that patients with higher doses of methylprednisolone were at risk of HCMV disease at lower viral loads. This data is somewhat surprising since an impaired immune response may be expected to increase virus replication due to lack of immune surveillance by T-cells. With impaired T-cell function, an alternative mechanism of NK killing may predominate to control systemic virus replication. Both the mouse and rat model for CMV have demonstrated the importance of NK cells at controlling virus replication in the absence of an intact T-cell mediated immune response (Lathbury *et al*, 1996, Van Dam *et al*, 1997). Data from these two studies revealed that NK cells were predominantly responsible for controlling virus replication when the T-cell mediated immune response was depleted. Lathbury *et al* selectively reduced CD4+, CD8+ and NK cells in mice using monoclonal antibodies and investigated the level of MCMV replication in various organs. The results from this study revealed that in the absence of CD8+ T-cells, virus replication was controlled by NK cells. In the later study by Van Dam *et al* (1997) the numbers of NK cells increased markedly in immunocompromised rats following infection with RCMV. Specifically, increased numbers of infiltrating NK cells were detected in all visceral organs in association with increased viral titres. In contrast, the number of NK cells and viral titre in the salivary gland, a known site of latency for MCMV were significantly reduced. It is important to note however, that these data may not necessarily reflect the complex series of events that take place in the human system.

To allow viral persistence and immune evasion, HCMV possess a variety of genes including the class I homologue UL18. During infection, NK cells recognise virus infected cells that do not display MHC class I, in this instance UL18 can act as an NK decoy to avoid NK attack (Farrell *et al*, 1997, Reybum *et al*, 1997). This further confounds the data presented in chapter 5 of increased probability of HCMV disease with reduced HCMV viral load in the presence of increased augmented methylprednisolone. With an immune

evasion mechanism in place and an impaired T-cell mediated immune response, one would expect virus replication to increase. However, the pathology caused by HCMV at a localised organ site may only require limited virus replication to cause tissue damage which may not be visible to circulating NK cells or residual T-cells. It is possible that the effect of methylprednisolone at reducing the numbers of macrophages is important to allow organ specific pathology at lower viral load levels. Alternatively, the ability of NK cells to reduce pathology is overwhelmed at lower viral load levels. In order to address these issues further studies involving HCMV quantitation in organ biopsies together with *in situ* hybridisation and PCR will be necessary.

In chapter 6 of this thesis the dynamics of the humoral immune response to HCMV was investigated following liver transplantation. The results obtained for total IgG responses provided little information with respect to the detection of systemic HCMV. In contrast, the dynamics of the specific anti-gB antibodies were significantly related to the detection and level of HCMV DNA in blood. Indeed, there was a trend for HCMV DNA detection to occur prior to the first significant rise in anti-gB antibody levels, while peak HCMV viral load was detected significantly earlier than peak anti-gB antibody levels. The level of anti-gB antibodies was also significantly associated with the level of HCMV viral load in blood ($p=0.0007$). These data imply that the B-cell specific immune response to HCMV is mediated by the level of systemic viraemia in the immunocompromised host. It would be interesting in future studies to analyse the timing of this specific anti-gB antibody response in relation to CD8+ specific CTL and CD4+ T-cell frequencies together with the levels of HCMV DNAemia. These data may allow the relative contributions of the B and T cell components of the immune system at controlling HCMV infection to be assessed.

Having established a relationship between the detection of HCMV DNAemia and the specific anti-gB antibody response, the effect of the serostatus of liver transplant recipients on this relationship was also analysed. In a recent report by Schoppel *et al* (1996), immunocompromised transplant recipients at risk of primary HCMV infection experienced a delay in the production of glycoprotein specific antibody levels, whereas in recipient seropositive individuals a synchronised production of these antibodies was found. These data are in agreement with the results obtained from the time dependent analysis carried out for liver transplant patients at risk of either reactivation or reinfection or primary HCMV infection presented in chapter 6. However, unlike the study by Schoppel *et al*, the results presented here also examined the levels and timing of specific anti-gB

antibodies in relation to the level and timing of HCMV viral load. There was a significant delay in the detection of both initial and peak anti-gB antibodies in relation to the first detection of HCMV DNAemia and peak viral load in patients with primary HCMV infection compared to HCMV recipient seropositive patients. Indeed, HCMV seropositive patients had an initial anti-gB antibody response at a median of 3.5 days following the first detection of HCMV DNAemia compared to 10 days in recipient seronegative patients ($p < 0.05$). Similarly, peak anti-gB antibody levels were detected significantly earlier in HCMV seropositive patients versus seronegative patients equivalent to a median of 14 days.

It is assumed, in the case of HCMV that the T-cell mediated immune response is required to suppress virus replication. However, the pathogenesis of HCMV following establishment of latency or during reactivation as a result of immunosuppression in the immunocompromised host remains to be fully elucidated. Indeed, in the murine model it has been shown that antibodies in primed mice are important to limit virus dissemination to a wide variety of organs (Jonjic *et al*, 1994). It has also been shown for MCMV that the level of virus distributed to organs during primary infection, can also influence the incidence of recurrence at that same site (Reddehase *et al*, 1994). By extrapolation of these data to the human system, it is interesting to speculate that patients with primary HCMV infection who have an increased risk of HCMV disease and significantly higher viral loads in blood as a result of multiple organ involvement, may be due to the delay in neutralising antibody levels to control replication in the absence of an intact T-cell mediated immune response. In contrast, in patients with a primed immune response where the synthesis of potentially neutralising antibodies appears significantly earlier, viral dissemination is restricted leading to lower viral loads in blood and consequently a reduced incidence of HCMV disease.

The results presented in this thesis clearly demonstrate the utility of qualitative and quantitative PCR assays to provide prognostic information for HCMV disease. However, the detection of HCMV DNA does not allude to the patterns of gene expression. At present, the prognostic value of measuring mRNA levels in immunocompromised patients remains to be fully elucidated. Consequently, I developed a fully quantitative competitive reverse transcriptase PCR assay (QCRT-PCR) for the detection of gB mRNA of HCMV (Chapter 7) This information may be of great importance when assessing antiviral efficacy or levels of gene expression in specific cell types to provide information into the pathogenesis of HCMV. Indeed, the use of RT-PCR has led to the identification

of specific cell types that may act as a reservoir for latency following HCMV infection (Fish *et al*, 1995, Kondo *et al* , 1996, Movassagh *et al*, 1996, Kurz *et al* , 1997, Zhuravaskaya *et al*, 1997). Application of the QCRT-PCR assay in chapter 7 revealed that maximum levels of gB mRNA were reached at 24 hours post infection which contradicts the previous identification of the gB gene as a late structural gene product. However, a more recent report by Smuda *et al* (1997), identified a series of transcripts that were produced at early and late times from the UL54/UL55 gene block encoding HCMV DNA polymerase and HCMV gB respectively. The expression of the larger 8kb transcript appeared at the early times (12-24 hours post infection), which would account for the maximum levels of gB mRNA detected at 24 hours, while the smaller transcript was detected at later times (36 hours pi), which would account for the detection of gB mRNA as late as 96 hours pi.

In the clinical setting there is limited data relating the utility of HCMV mRNA levels in the context of HCMV disease and response to antiviral therapy. The majority of previous studies have relied on the qualitative detection of mRNA from the IE genes of HCMV with poor sensitivity and specificity. Patel *et al* (1995) compared the use of cell culture and the detection of HCMV DNA by PCR with RT-PCR for the early detection of HCMV in 41 liver transplant recipients. The results from this study showed that RT-PCR for detection of IE mRNA only detected 25% of symptomatic patients. However the specificity of the assay was high (97%) compared to only 45% and 35% for DNA PCR of serum and blood culture respectively. In a later study by Gozlan *et al* (1996), RT-PCR the detection of mRNA from the late MCP gene of HCMV in the blood of 45 bone marrow transplant recipients was analysed. The sensitivity and specificity of this RT-PCR assay was 71% and 94% respectively. These data illustrate the importance of the choice of mRNA as a target for the RT-PCR to provide prognostic information. It should also be noted that in both studies, only single sampling was available which would further confound the ability of the assay to provide prognostic information. The data presented in chapter 7 reveal the possibility of simultaneous detection of an early and a late gB transcript of HCMV. Consequently, this assay may have the advantage over previous assays of detecting more than one species of mRNA (both of which reflect active viral replication), thus improving both sensitivity and specificity. Further studies to evaluate the utility of this assay in the clinical setting are required to establish whether it could be used as a prognostic marker for HCMV disease by the establishment of threshold levels as is postulated for the levels of HCMV DNA viral load. Furthermore QCRT-PCR with its broad dynamic range may prove more appropriate to assess levels of gene expression

and response to antiviral therapy. Indeed, the use of NASBA based assays for the detection of mRNA from other viruses including HIV, HBV and HCV has proved to be of great importance in defining the pathogenesis of the associated diseases as well as determining antiviral efficacy. Indeed, a recent publication has assessed the use of a NASBA assay for the detection of the late gene pp67 of HCMV in renal transplant recipients with encouraging results (Blok *et al* 1998).

In conclusion, this thesis has provided important information on the pathogenesis of HCMV in the immunocompromised host by application of modern molecular biological techniques. It has identified HCMV viral load as critical in the context of HCMV disease and has allowed an appreciation of other risk factors which are both dependent and independent of viral load. The application of these concepts to the immunocompromised individual will hopefully allow further reductions in the incidence of HCMV disease.

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List of Publications resulting from the work in this Thesis

1. Bowen EF, Wilson P, Cope A, *et al.* Cytomegalovirus retinitis in AIDS patients: influence of cytomegaloviral load on response to ganciclovir, time to recurrence and survival. *AIDS* 1996; 10: 1515-1520.
2. Cope AV, Sweny P, Sabin C, *et al.* Quantity of cytomegalovirus viraemia is a major risk factor for cytomegalovirus disease after renal transplantation. *Journal of Medical Virology* 1997; 52:200-205
3. Cope AV, Sabin C, Burroughs A, *et al.* Inter-relationships between quantity of human cytomegalovirus DNA in blood, donor/recipient serostatus and administration of methylprednisolone as risk factors for HCMV disease following liver transplantation. *Journal of Infectious Diseases* 1997;176(6):1484-90.
4. Hassan-Walker AF, Cope AV, Griffiths PD, *et al.* Transcription of the human cytomegalovirus natural killer decoy gene UL18 in vitro and in vivo. *Journal of General Virology* (In Press).

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