STUDIES OF THE NATURAL HISTORY OF HUMAN CYTOMEGALOVIRUS INFECTION IN THE HIV INFECTED HOST.

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by

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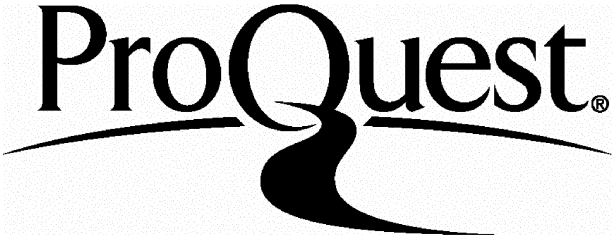

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ABSTRACT

This thesis examined the relationship between human cytomegalovirus (HCMV) infection and HCMV disease occurrence in patients co-infected with HIV. HCMV PCR was used to screen asymptomatic patients with low CD4 counts for their risk of developing HCMV disease. Patients who were HCMV PCR positive had a 21 fold increase in risk of HCMV disease compared to those who remained HCMV PCR negative. I suggest that PCR positive patients are a high risk group in whom controlled trials of pre-emptive therapy should be conducted.

The quantity of HCMV present in blood (HCMV load) at the time of diagnosis of retinitis was found to have important implications. Firstly, if left untreated HCMV load continued to increase, HCMV retinitis progressed and HCMV disease occurred in other organs. Secondly, patients with an HCMV load >5 $\log_{10}$ genomes/ml blood at diagnosis of retinitis responded less well to ganciclovir induction therapy and had a shorter time to first progression of retinitis. Thirdly, there was a significant association between high HCMV loads at retinitis presentation and reduced survival.

Whilst receiving maintenance therapy 80% of patients had at least one episode of retinitis progression which occurred mostly in the absence of detectable PCR-viraemia. I suggest this was likely to be due to local reactivation of virus in the retina. Patients who did become PCR positive during maintenance therapy all had an episode of retinitis progression and had a significantly higher risk of developing other HCMV disease. Patients who were PCR positive were also more likely to develop mutations in the UL97 gene conferring ganciclovir resistance. I developed a point mutation assay for screening patient samples for the presence of the five most common UL97 resistant mutations. These results were then used to study the viral fitness of different UL97 mutants and hence, estimate the *in vivo* generation time of HCMV. I expressed one of these UL97 mutants (L595F) in a recombinant baculovirus expression system in preparation for functional studies.

All studies conducted in this thesis had gained full approval from the Royal Free Hospital School of Medicine Ethics Committee.

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TO MY MOTHER AND FATHER

TREFOR LLEWELLYN BOWEN

FRCS

1932 - 1974

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ABBREVIATIONS

aa	amino acid
AI	adrenal insufficiency
AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
AZT	3' azidothymidine
b.d	bis die (twice a day)
bp	base pairs
BSA	bovine serum albumin
CDC	Centre 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
GCSF	granulocyte colony stimulating factor
GCV	ganciclovir
GID	gatrointestinal 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
Ig	Immunoglobulin
IT	induction therapy
iv	intravenous
kb	kilobase
LTR	long terminal repeat

M	matrix protein
mAb	monoclonal antibody
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	*pneumocystis carinii* pneumonia
PCR	polymerase chain reaction
PFA	foscarnet
pfu	plaque forming unit
pi	post-inoculation
PM	post mortem
PMA	point mutation assay
po	per oram (orally)
pp	phosphoprotein
QCPCR	quantitative competitive 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
Taq	*Thermus aquaticus*
TB	tuberculosis
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

1.0 HUMAN CYTOMEGALOVIRUS

1.1 Morphology

Human cytomegalovirus (HCMV) is a member of the family *Herpesviridae* which has three recognized sub-groups, *alpha, beta, and gamma herpesvirinae.* HCMV is classified as a member of the *betaherpesvirinae* based on its narrow host range, slow *in vitro* growth and its classic cytopathic effect of cytomegalia (Honess and Roizman, 1975). Indeed, it was the observation of this classical cytomegalic effect on infected cells that led Weller in 1957 to name the virus Cytomegalovirus (Weller *et al.* 1957). The morphology of HCMV is typical for all herpesviruses with the central DNA core surrounded by a capsid made up of 162 hollow hexagonal capsomers. The capsid is covered by a poorly demarcated tegument of phosphoproteins and an outer envelope thought to originate from the internal nuclear membrane (Emery and Griffiths, 1990). The outer envelope is a lipid bilayer through which the virally encoded envelope glycoproteins protrude.

1.2 The Genome

The HCMV genome, the largest of the herpesviruses described, is a double-stranded DNA virus made up of 229,354 base-pairs, in laboratory strain Ad169, divided into two unique regions termed unique long (UL 169,972 bp long) and unique short (US 35,418 bp long). UL is flanked by two repeat sequences called inverted and terminal repeats (IR_L and TR_L respectively), while US is flanked by two repeats namely IRs and TRs (Mocarski, 1996). Each unique region can be present in either orientation thus resulting in four possible isomeric forms of virion DNA which occur in equimolar amounts. The genome has a relatively high G+C content (57.2%) although there is considerable variation in the G+C distribution throughout the genome. The genome of the laboratory strain Ad169 has been

sequenced in its entirety (Chee MS. and Bankier AT. 1990). The availability of this sequence on EMBL and Genbank databases has facilitated comparison of experimentally derived sequences from patient material with this "prototype" sequence. However, it should be remembered when extrapolating these data to clinical strains that Ad169 is a poorly pathogenic, highly passaged strain of HCMV adapted to cell culture and its relevance to *in vivo* strains of HCMV and patterns of clinical disease has yet to be fully elucidated. Indeed, it has recently been shown that HCMV clinical isolates carry at least 22 open reading frames (some of which were lost by UL rearrangement) not found in laboratory strains (Cha *et al.* 1996).

Multiple strains of HCMV co-exist *in vivo* and can easily be identified by enzymatic digestion with restriction endonucleases (Chou, 1990). These characteristic restriction fragment length polymorphisms (RFLPs) result from mostly silent point mutations between different strains. Darlington *et al* used restriction endonuclease analysis, polymerase chain reaction (PCR) and sequence analysis to study the glycoprotein B (gB) region and found nucleotide changes in approximately one third of strains of which 10% were coding changes (Darlington *et al.* 1991). Although identical RFLPs cannot prove that two strains of HCMV are identical, they can be used to study the source of infection and viral pathogenesis in immunocompromised patient groups.

1.3 Proteins

The genome is large enough to encode over 220 proteins and sequencing by Bankier *et al* has identified 204 predicted open reading frames (Bankier *et al.* 1991). However, due to duplication within repeat regions and known splicing events, there are approximately 178 unique proteins in Ad169 (Griffiths and Emery, 1996). Approximately 30 genes, which are

present in the UL region, are likely to be essential genes as they are conserved between the three *Herpesviridae* sub-families. The genes are numbered according to their relative position in UL or US. The proteins they encode are then designated by *p* (protein), *gp* (glycoprotein) or *pp* (phosphoprotein) followed by the gene number (Landini Elsevier). Figure 1.1 shows a schematic illustration of the HCMV genome. Any preferred trivial name may be given in brackets after the position on the genetic locus. This introduction will concern itself only with the genes and proteins relevant to this thesis and a comprehensive review of other proteins can be found by Mocarski in 'Cytomegaloviruses and Their Replication', Fields Virology 1996 (Mocarski, 1996).

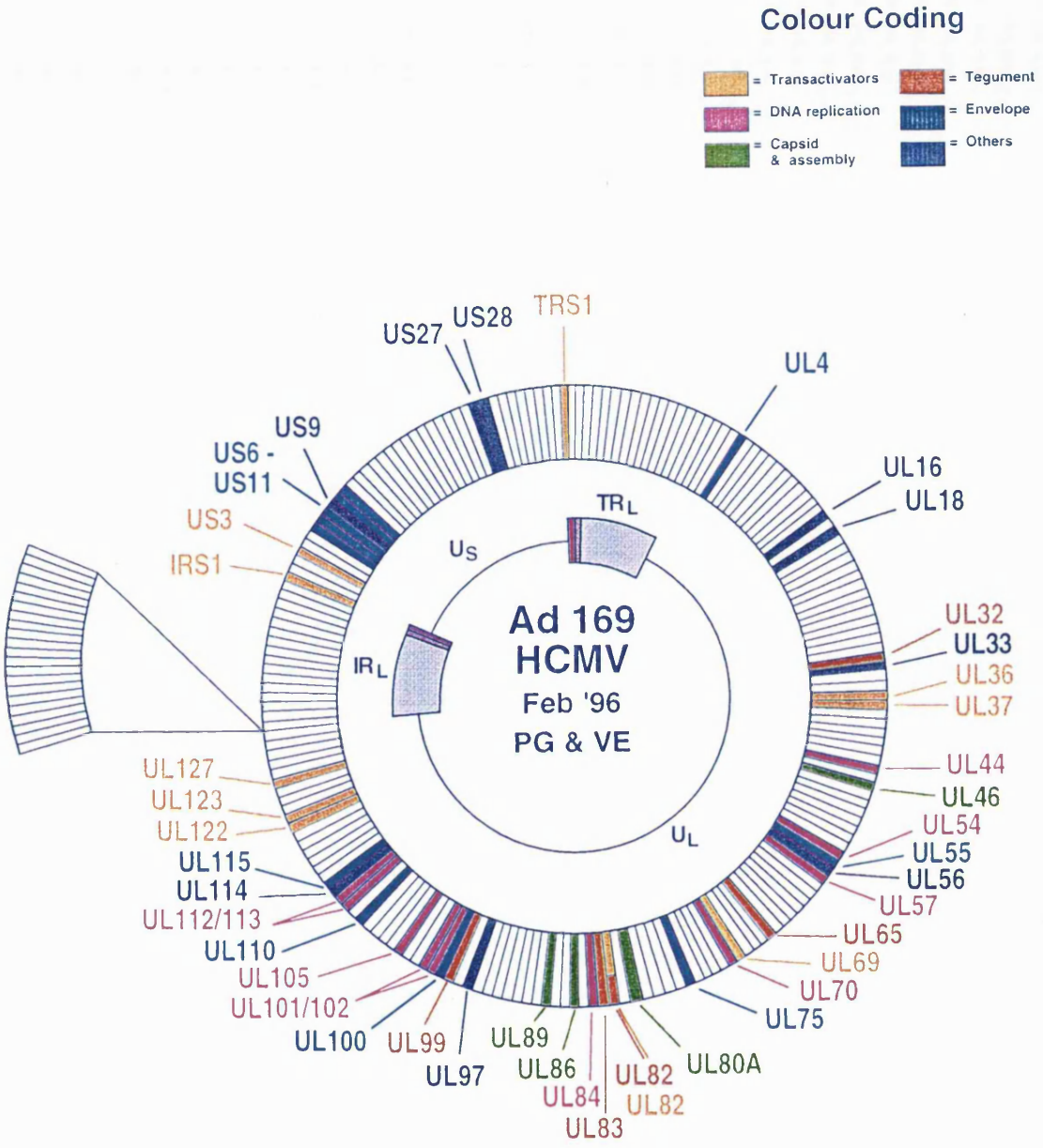


Figure 1.1: Organisation of the HCMV genome (kindly reproduced with permission from Professor P. Griffiths and Dr V. Emery).

1.4 Regulation of Gene Expression

Expression of the HCMV genome is controlled by a cascade synthesis of proteins very similar to that originally described by Roizman for herpes simplex virus (HSV) replication (Roizman and Furlong, 1974). The cascade for HCMV consists of sequential expression of immediate-early (IE or α), early (E or β), and late (L or γ) proteins. The IE genes are the first to be transcribed within 2 hours of virus entry into the host cell. IE expression requires no prior viral protein synthesis and is initiated by host cell RNA polymerase II. The major IE region maps to UL122/123 which contains upstream enhancers and is a strong regulatory region. pUL69 acts in synergy with ppUL82 to activate the major IE (MIE) promoter. Subsequently the transcription of the viral messenger RNA (mRNA) for the E proteins occurs, viral DNA replication proceeds and the L genes are then expressed. Additionally, the synthesis of many cellular proteins (including DNA and RNA polymerases, plasminogen activator and DNAses) is increased after infection with HCMV suggesting a possible requirement for some of these cellular enzymes in viral replication (Albrecht *et al.* 1989). Analysis of the cellular DNA of HCMV infected cells shows a disruption of the cell cycle with a progressive shift of the cells into S phase in association with the over expression of cyclin E and p53 (Jault *et al.* 1995).

1.5 DNA Replication

Viral DNA synthesis occurs at a slower rate than for the alpha herpesviruses with two peaks of HCMV DNA replication found at one to three days post-infection (pi), compared to 4-6 hours for HSV. This is borne out by the appearance of HCMV plaques in cell culture at 7-14 days, about one week later than the time taken for HSV plaques to develop (Mocarski, 1996). HCMV uses rolling circle replication resulting in concatameric

molecules that are cleaved into unit length genomes during packaging into virions. Human CMV DNA synthesis utilizes both a *cis*-acting replicator (*ori*Lyt) and *trans*-acting viral proteins (Anders and Punturieri, 1991). A total of 11 genes, encoding proteins in 11 distinct loci, are required for HCMV *ori*Lyt-mediated DNA replication (Pari and Anders, 1993). Six of these defined loci encode proteins common in the herpesvirus group and their roles in HCMV DNA synthesis are predicted according to their homology with HSV-1 replicatory genes. These include the HCMV DNA polymerase (UL54, *pol*), a Mr 140,000 protein, similar to other herpesvirus polymerases in that it has 3'-5' exonuclease activity, is active in high salt concentrations and is sensitive to deoxyribonucleoside analogues (Mocarski, 1996). Human CMV DNA polymerase is also found in association with HSV analogue ICP36 (UL44 gene product), a 51-58 kDa protein that has been shown to bind to HCMV DNA *pol* and stimulate enzymatic activity (Ertl and Powell, 1992). The remaining four proteins of the 11 required include the single stranded DNA-binding protein (pUL57) and three proteins (pUL70, pUL102, and pUL105) homologous to HSV-1 helicase-primase subunits (Iskenderian *et al.* 1996). The other loci encode IE regulatory proteins (UL36-38 and UL122-123 from the MIE region) and proteins of unknown function (UL84, UL112-113) all of which are required to complement the expression of the other replicatory genes (Iskenderian *et al.* 1996). A terminase complex is associated with the packaging and cleavage of concatamers. This is thought to involve the gene UL89 as resistance to a new antiviral agent (bromodichloro benzimadazole riboside (BDCRB), inhibits the cleavage of DNA intermediates to monomeric units and thus prevent the formation of infectious virus particles) has been located to UL89.

1.6 Virion Packaging

It is the expression of the L proteins that allows the assembly of mature virions to occur. This begins 72 hours pi with the accumulation of nucleocapsids in the nucleus which gives rise to the classic "owl's eye" cytopathic effect. The nucleocapsids are thought to be formed by a distinct pathway where they mature within a network of viral structural proteins and DNA (Fons *et al.* 1986). HCMV capsids are formed prior to DNA packaging and the envelope is thought to be either acquired from the inner nuclear membrane or the virion may lose and then re-acquire its envelope from endosomes during egress (Tooze *et al.* 1993). The virion may also be transported in vesicles via the Golgi apparatus to the cell surface and egress in an exocytic manner. Structurally complete virions are released into the extracellular medium by exocytosis by 120 hours pi. Structural proteins of the virus include the major capsid protein UL86 and the minor capsid protein UL46. UL80a is the assemblin gene complex and contains a viral protease as well as assembly protein for DNA packaging (Holwerda *et al.* 1994). The three dimensional structure for the HCMV protease has recently been published showing that the HCMV protease has a single domain which is a seven stranded β-barrel core encircled by seven α-helices (Qiu *et al.* 1996). The crystal structure reveals an active-site triad consisting of a serine at position 132 flanked by two histidine residues at positions 63 and 157. It is thought that dimerisation of the protease is necessary for activity (Margosiak *et al.* 1996) and dimerisation has been seen in the crystals (Qiu *et al.* 1996). In addition to the mature virions, two types of defective particles are also seen *in vitro* studies with Ad169. Non-infectious particles (NIEPs) are morphologically indistinguishable from mature virions but lack genetic material. The second type are dense bodies which are much larger particles made up of a mass of tegument protein consisting predominantly of ppUL83 (pp65). The significance of these particles *in vivo* has yet to be

fully elucidated.

1.7 Epidemiology

Human CMV is species specific and humans are the only reservoir. Fifty to 100% of the adult population have been exposed to HCMV as judged by the presence of IgG antibodies specific to the virus. This figure varies according to important factors such as socio-economic group, closeness of contacts and hygiene. Therefore, approximately 60% of adults in the UK are HCMV seropositive compared to 90-100% of adults in developing countries (Britt and Alford, 1996). Sexual activity is important in the transmission of HCMV as indicated by the high seroprevalence among patients attending sexual health clinics (Shen *et al.* 1994). Homosexual males have a greater risk of acquiring HCMV infection than heterosexual males (Berry *et al.* 1988) and over 95% of homosexual men have been found to be seropositive for HCMV (Drew, 1988). Indeed, in homosexual men genital shedding of HCMV is increased in both frequency and quantity of virus in comparison to heterosexual men (Mintz *et al.* 1983). HCMV is perpetuated through the community by being excreted asymptomatically from many sites, including most mucous membranes and in all body fluids (saliva, urine, blood, semen, vaginal secretions and breast milk). Spread can therefore be achieved both horizontally to close contacts and vertically to the fetus/neonate. Indeed, intrauterine HCMV infection is the commonest cause of symptomatic congenital infection in the UK and may lead to severe fetal damage (Fowler *et al.* 1992). Horizontal transmission is facilitated by the persistent sub-clinical shedding of HCMV in the urine of healthy young children in kindergartens (Shen *et al.* 1993). This shedding has been characterised in one study, using restriction enzyme digestion of PCR amplified gB, as being persistent excretion of endogenous virus rather than reinfection with

another strain of HCMV (Shen *et al.* 1996).

After the neonatal period, acquisition of HCMV in the immunocompetent host is asymptomatic in over 90% of cases and manifests as an infectious mononucleosis type syndrome in the remaining cases that may often be labelled as Epstein Barr Virus (EBV) infection. However, in the immunocompromised host, primary HCMV infection, usually via a donor organ or, to a lesser extent, blood transfusion, is a major cause of morbidity and mortality. Primary HCMV infection acquired from blood transfusion (the postperfusion syndrome) is thought to be due to latent HCMV within leucocytes that reactivates on encountering an allogeneic stimulus in the transfusion recipient (Winston *et al.* 1980). Therefore, measures are taken to give HCMV negative blood products for HCMV seronegative transplant recipients. By far the commonest cause of HCMV disease in the immunocompromised host is due to recurrent HCMV infection either from reactivation of endogenous latent virus or reinfection from exogenous virus.

1.8 Pathology

The end-organ damage seen in HCMV infection of the gastrointestinal tract has been studied closely. On a macroscopic level, damage is due to vascular infection and inflammation leading to focal thrombosis, infarction and destruction of the area of bowel supplied with blood (Meiselman *et al.* 1985). A similar sequence of events is thought to occur in HCMV mediated destruction of the adrenal gland. This is in contrast to the HCMV pneumonitis seen in BMT recipients which is thought to be an immunopathologically mediated condition. As pneumonitis is not seen in syngeneic transplants one theory is that the damage is from T cell responses against virally induced antigens on the hosts alveolar cells (Grundy *et al.* 1987). In the case of HCMV retinitis, the exact mechanism of how

HCMV infects and subsequently destroys the retina remains unclear.

After primary infection, HCMV establishes latency in the infected host in many sites although it is not fully understood which cells/tissues are the reservoir of latent virus. Human CMV has been shown to persist in monocytes both by *in situ* hybridisation and the amplification of viral DNA from fractionated cell populations (Taylor-Wiedeman *et al.* 1991). It has been shown in an *in vitro* monocyte derived macrophage culture system that productive HCMV infection is significantly delayed compared to HCMV production in fibroblasts, and persists in these cells without lytic events (Fish *et al.* 1995). Indeed, during late stage infection, HCMV is recovered from vacuoles that are thought to be derived (due to the presence of mannosidase II) from the Golgi apparatus (Fish *et al.* 1996). These virus containing vacuoles then evade lysosomal fusion (by HCMV induced destruction of the microtubule network) and prevent their transport to the plasma membrane. The authors postulate that this sequestration of virus in vacuoles serves two purposes; firstly, to protect virus from cellular degradation and protect the cell from viral protein induced damage. Secondly, the virus is hidden in intracellular compartments that do not reach the plasma membrane and, therefore, escapes surveillance and clearance by the immune system (Fish *et al.* 1995).

Human CMV can also mediate its own 'escape' from the immune system by selectively modifying the expression of certain viral proteins and the effectiveness of the host immune response. Human CMV can itself greatly influence the clearance of virus by down regulating the expression of MHC class 1 proteins. It has been shown *in vitro* that 3 days pi the level of class 1 expression on the surface of HCMV infected fibroblasts was only 50 % of that on uninfected cells (Grundy and Downes, 1993). This HCMV mediated down-

regulation of MHC class 1 heavy chains may occur due to interference with the stability of class 1 heavy chains rather than affecting class 1 heavy chain mRNA synthesis (Beersma *et al.* 1993). More detailed molecular studies have confirmed that the down regulation of MHC class I begins at 3 hours pi and continues throughout the infectious cycle. The deletion of a 7 kb fragment in the US region of the HCMV genome results in the loss of wild type down regulation phenotype (Jones *et al.* 1995). Further marker transfer studies identified that US11 encodes a glycoprotein that can down regulate class I heavy chains in the absence of other viral gene products (Wiertz *et al.* 1996). The US11 gene product encodes an endoplasmic reticulum (ER) based transmembrane glycoprotein that allows class I heavy chains to move from the ER into the cytosol where they are rapidly degraded (Wiertz *et al.* 1996). Concomitant with the reduction in MHC class I expression, HCMV induces the expression of adhesion molecules LFA-3 and ICAM-1 that may significantly alter leukocyte tracking during HCMV infection (Grundy and Downes, 1993). Gilbert *et al* have shown that pp65 expression results in the phosphorylation of IE in threonine residues that subsequently interferes with IE presentation without significantly altering either the stability of the IE protein or its ability to activate transcription (Gilbert *et al.* 1996). Polymorphonuclear cells are not thought to be a source of latent HCMV (Taylor-Wiedeman *et al.* 1991). Evidence of HCMV infection can be found widely distributed throughout many organs in both immunocompetent individuals where IE antigen expression was detected at post-mortem in brain, kidney, lung, spleen and liver (Toorkey and Carrigan, 1989) and in patients with immunosuppression secondary to HIV infection where HCMV was detected at post mortem in 66% and 100% of patients using cell culture and PCR respectively (Pillay *et al.* 1993; Webster *et al.* 1995).

1.9 Murine Models of CMV Pathology

There is no clear explanation as to why HCMV reactivates in different organ systems under different conditions and at different times. This question has been addressed in the murine model where the risks of recurrence of murine CMV were compared in experimental mice infected either as neonates or as adults (Reddehase *et al.* 1994). Mice infected neonatally had a high mortality with primary infection and widespread high level viral replication in many organs. The survivors subsequently had a higher risk of recurrence than mice whose primary murine CMV (MCMV) infection occurred as adults where high titres of viral genomes were only detected in the lung. Therefore, it appeared that latent MCMV burden and the risk of recurrence were related to the degree and dissemination of MCMV replication during primary infection (Reddehase *et al.* 1994). Murine models have also been used to study CMV infection of the retina. One hypothesis, that HCMV infection seeds the retina through pathways other than the retinal capillaries, has been supported with an animal model using Balb/C nude mice (Hayashi *et al.* 1995). In these mice MCMV first reached the conjunctiva and then spread to the outer ocular muscles and then to the chorioretinal layer. However, although animal viruses have similar disease processes to our human counterparts, HCMV is very host specific and therefore the relevance of data generated in animal models of CMV in the pathogenesis of HCMV retinitis should be treated with caution. Diloreto *et al* have, however, established an *in vivo* system for growing human fetal retina in immune deficient mice (DiLoreto, Jr. *et al.* 1994). In early studies the grafts became well established histologically and histochemically by 60 days. HCMV infected grafts showed the typical inclusions and HCMV was recovered from one graft 45 days pi and successfully passaged in fibroblast culture.

Athymic nude mice have been used to study the pathology of adrenal destruction with MCMV infection. In the absence of T-lymphocyte function MCMV infects and systematically destroys both the adrenal medulla and cortex (Shanley and Pesanti, 1986). This acute infection could be abrogated by the adoptive transfer of MCMV-immune, but not naive, spleen cells (Shanley, 1987). The presence of competent MHC class II restricted T-lymphocytes protected the adrenal gland from destruction for up to 60 days. These early studies highlight the importance of both T cell competence and the predilection of CMV for the adrenal gland.

1.10 Pathogenesis

Following HCMV infection, virus spreads to a variety of organs by viraemia and particularly infects ductal epithelial cells. This is often associated with viral replication in the genitourinary tract and is reflected by asymptomatic viruria. Circulating cytomegalic inclusion cells of endothelial origin can be detected in the peripheral blood of individuals with acute HCMV infection (Grefte *et al.* 1993). There is evidence that peripheral blood mononuclear cells and endothelial cells *in vitro* can transmit HCMV in a bidirectional manner. This important interface between infected peripheral blood cells and the endothelium may play a pivotal role in the widespread tissue dissemination of HCMV during acute infection. Particular HCMV strains may be more pathogenic in that viraemia caused by gB group 2 HCMV strains is associated with a higher incidence of retinitis than viraemia due to other HCMV gB groups (Shepp 1996).

In the immunocompetent host both cellular and humoral immune responses are involved in generating immunity to HCMV. Other aspects of the immune response that are thought to be important in this protective immunity are the mucosal immune response and cytokines.

However, as noted by Britt (in Fields), all of these observations have been made in specific groups unable to raise a particular response to HCMV infection and are therefore, examples of the immune response at a particular time point of infection rather than a definition of the entire protective mechanism (Britt and Alford, 1996).

1.11 The Host Immune Response to HCMV

1.11.1 Cellular Immune Response

A cellular immune response has been demonstrated following acute HCMV infection and involves MHC class 1 restricted cytotoxic T lymphocytes (CTL), antibody dependent cellular cytotoxicity (ADCC) and natural killer cells (Britt and Alford, 1996). Riddell *et al* demonstrated that the HCMV specific class-I restricted CTL response was specific for structural virion proteins introduced into the cell after viral penetration (Riddell *et al.* 1991). This response has more recently been shown to be mostly directed to the structural protein pp65 (pUL83). A pp65 HCMV deletion mutant was used to estimate the contribution of pp65-specific CTL's to the total HCMV CTL-specific immune response (Wills *et al.* 1996). Between 70 and 90% of all CTL's recognising HCMV infected cells were pp65 specific. Moreover, four of the patients studied shared the HLA A2 allele and all recognised the same pp65 peptide. The generation of a specific CTL response is becoming increasingly recognised as playing a very important role in the post-transplant period, either naturally via the hosts own immunity, or adoptively when infused passively. In three HCMV seropositive bone marrow donors CD8+ HCMV specific CTL clones were generated and propagated *in vitro* for 5-12 weeks and then infused into the BMT recipient weekly for four weeks (Riddell *et al.* 1992). Adoptively transferred CD8+ CTL immunity was detectable in the peripheral blood up to four weeks post infusion. None of the three patients who received the CTL

infusions developed HCMV viraemia or pneumonitis in this uncontrolled study, suggesting that CD8+ CTL responses may be able to confer protection to HCMV disease (Reusser *et al.* 1991). Further understanding of the relationship between particular pp65-HLA class I associations could greatly improve the post-transplant management of patients with "high risk" HLA types by adoptively enhancing HCMV-specific CTL immunity. Further supporting evidence for the essential role of HCMV CTL's was found in neonates infected with HCMV in utero where infants with depressed CTL proliferative responses continued to excrete virus (Pass *et al.* 1983). The virally encoded targets of this specific CTL response remain to be fully elucidated although several proteins that are known to induce cellular responses are strong candidates. These include protein products of the IE-1 gene and structural proteins such as pp65 and pp150 (Britt and Alford, 1996). Another important aspect of the cellular immune response is the activation of non-specific natural killer cells which have been demonstrated in bronchoalveolar lavage (BAL) aspirates from bone marrow transplant patients (Bowden *et al.* 1986).

1.11.2 Humoral Immune Response

The importance of the antibody response to HCMV infection was first identified in neonates (infected post-natally by blood transfusions) where transplacentally acquired anti-HCMV antibody significantly reduced morbidity and mortality in premature infants. Furthermore, in congenital infection, the presence of maternal anti-HCMV antibody prior to conception has been shown to reduce subsequent fetal infection with HCMV (Fowler *et al.* 1992). In allogeneic, HLA matched, T-cell depleted bone marrow transplant recipients, bone marrow from HCMV sero-positive donors gave a protective effect against severe HCMV disease in sero-positive recipients (Grob J. and Prentice H. 1987). Patients who

received marrow from HCMV sero-negative donors had a fifty-fold greater risk of fatal HCMV infection. These findings have now been extrapolated to the clinical setting where both pooled and specific immunoglobulin are used in the prophylaxis of HCMV disease in bone marrow allograft and renal transplant recipients.

Part of the antibody mediated immune response is thought to involve neutralising antibodies to limit the dissemination of virions throughout the body during systemic disease. Two envelope glycoproteins UL55 (gB) and UL75 (gH) have been shown to strongly induce virus-neutralising antibodies. Antibodies to gB and gH were detected at different frequencies in immunocompetent individuals after natural HCMV infection. In one study, antibodies to gB were often detected but antibodies to gH were rarely detected unless there was a recent history of acute symptomatic HCMV seroconversion, leading the authors to suggest that some individuals may fail to produce antibodies to gH in cases of asymptomatic primary HCMV infection (Rasmussen *et al.* 1991). The relationship between levels of these antibodies and the development of clinical disease is not fully understood. When comparing the antibody titres of gB and gH in AIDS patients, with and without HCMV retinitis, levels of anti-gB antibodies were elevated at levels comparable to HIV seronegative individuals with HCMV mononucleosis but antibodies to gH were rarely detected (Rasmussen *et al.* 1994). There were no measurements of the neutralising ability of these antibodies directed to either gB or gH. This is in contrast to another study of AIDS patients with HCMV retinitis where there was no evidence for a deficient antibody response to gB or gH in patients who had developed retinitis but there was a significant correlation between high levels of neutralising antibodies and slow progression of HCMV retinitis as determined by reading of masked retinal photographs (Boppana *et al.* 1995). The majority of neutralizing antibodies are directed against gB which has at least two dominant binding sites; AD-1 and

AD-2. In a recent study comparing the sequence variation of AD-1 in patients with AIDS and renal transplant recipients there were a number of coding mutations found in the AD-1 domain even though these mutations did not appear to affect neutralisation activity (Roy *et al.* 1993). The number of mutations in AD-1 was much greater in the AIDS patients than the renal transplant recipients.

1.12 HCMV Infection in the Transplant Recipient

Patients undergoing transplantation can have their risk for HCMV disease assessed according to the HCMV serostatus of donor and recipient. For example, a seronegative recipient of a seronegative donor kidney has a very low risk of acquiring an HCMV primary infection compared to 60-80% of cases where a seropositive kidney is transplanted into a seronegative recipient (Griffiths, 1995b). Using RFLP strain typing HCMV disease occurring post renal transplantation has been shown to be from reinfection of the host with HCMV "passenger" strains present in the donor kidney (Grundy *et al.* 1988). This is also thought to be the case in other solid organ transplants such as heart transplantation (Chou S. 1989). In contrast, in bone marrow transplant recipients, it is the reactivation of endogenous host derived strains rather than reinfection from the donor that is responsible for HCMV disease (Winston D J. *et al.* 1985). In the transplant setting, the onset of HCMV infection typically occurs 3 to 6 weeks post transplantation and infection after this time, as immunosuppressive regimens are reduced, becomes less common. However, there are now incidences of HCMV disease occurring much later after transplantation once ganciclovir prophylaxis has been stopped (Goodrich *et al.* 1991).

HCMV infection is suspected clinically with high swinging fevers, leucopenia, elevation of hepatic transaminases and a falling platelet count. The spectrum of end-organ damage seen

with HCMV disease in the transplant recipient varies greatly according to the organ transplanted. In the iatrogenically immunosuppressed recipient of an allogeneic bone marrow transplant HCMV pneumonitis is the most common and severe manifestation of HCMV disease and is associated with a mortality of up to 80% (Meyers, 1986). This high mortality is more frequent when associated with graft versus host disease and is not seen in recipients of syngeneic transplants. Pneumonitis is seen much less frequently in renal transplant recipients where unidentified fevers, leucopenia and gastrointestinal disease are the most common manifestations of HCMV disease. Retinitis is much less common in renal transplant recipients. This is all in stark contrast to the pattern of HCMV disease in HIV positive patients where retinitis is by far the most common presentation of HCMV disease see Table 1.1.

Table 1.1: The spectrum of HCMV disease in different immunocompromised patient groups.

Symptoms	Solid organ Tx	Bone marrow Tx	AIDS
Fever/hepatitis	++	+	+
GI disease	+	+	++
Retinitis	+	+	+++
Pneumonitis	+	++	
Immunosuppression	+		
Myelosuppression		++	
Encephalopathy/ polyradiculopathy			+
Hypoadrenalism			+
Rejection/GVHD	?	?	

\+ <10% patients
++ 11-50% patients
+++ >51% patients

? = postulated but not proven association

1.13 Interactions Between HCMV and HIV

Human CMV exhibits bi-directional interactions with HIV in patients dually infected with both viruses. In co-infected cells in culture, HCMV and HIV can reciprocally enhance virus replication and co-infection has also been demonstrated *in vivo* in both brain and retina (Skolnik *et al.* 1988). Human CMV IE proteins have been shown to transactivate HIV LTR transcription, even in cellular pathways normally unable to induce HIV LTR, and therefore enhance HIV replication *in vitro* (Paya *et al.* 1991). Indeed, mRNA from the major IE gene has been detected in CD4+, CD8+ and CD19+ lymphocytes of HIV+ patients with active HCMV infection (von Laer *et al.* 1995). HCMV infection itself can also induce the expression of Fcγ receptors in the host cell and therefore, make cell types not usually susceptible to HIV liable to infection through uptake of Ig coated HIV virions (McKeating *et al.* 1990). These interactions *in vivo* have been termed cofactor interactions (Griffiths, 1995a).

The evidence for the more rapid progression of HIV in patients co-infected with HCMV has come largely from epidemiological studies. Webster *et al* found from a multivariate analysis of HIV+ haemophiliac men that being HCMV seropositive was associated with a significantly faster time to progression of HIV disease (Webster *et al.* 1992). This effect has been found to persist on follow-up (Sabin *et al.* 1995) but a similar analysis of a different haemophiliac cohort did not produce significant results (Rabkin *et al.* 1993). In children the presence of HCMV DNAemia confers a significantly higher mortality rate (67% vs 4%) and a significantly shorter survival time (43 months vs 65 months) in comparison to children with no evidence of active HCMV infection (Nigro *et al.* 1996). In another study, HIV positive men who were persistently shedding HCMV either in semen or in urine had a significantly higher rate of HIV disease progression than patients who were HCMV culture

negative (Detels *et al.* 1994). *In vivo,* HCMV and HIV can co-infect cells and post-mortem studies have shown the co-existence of these viruses in a wide variety of tissues (Webster *et al.* 1995)

1.14 HCMV Infection in AIDS Patients

Human CMV is the commonest and most severe viral opportunistic infection in patients with HIV infection. It is an AIDS defining illness according to the CDC classification (Centres for Disease Control, Atlanta, US; Table 1.2). Greater than 95% of homosexual men with HIV infection are seropositive for HCMV and excretion of HCMV in the semen is common (Collier *et al.* 1987). Post-mortem studies have shown that 90% of patients with AIDS had evidence of active HCMV infection at death and up to 40% had evidence of retinitis (Drew, 1988). Prospective analyses of over 2000 patients with HIV have estimated that the risk of developing HCMV disease at 1 year was 10% in patients with a CD4 count <100 cells/μl and 20% for those with a CD4 count <50 cells/μl (Gallant *et al.* 1992; Pertel *et al.* 1992). The median survival after a diagnosis of HCMV disease was 173 days and HCMV disease itself was an independent predictor of death (Gallant *et al.* 1992). It is unclear whether this latter association between HCMV disease and decreased survival in AIDS patients is due primarily to HCMV accelerating HIV immunosuppression or merely identifies patients with an increased degree of HIV directed immune dysfunction. Following the advent of anti-retroviral combination therapy and prophylaxis for opportunistic infections (OI), patients with HIV infection are living longer, particularly with more profound degrees of immunosuppression. This means that the incidence of HCMV disease has increased in patients with AIDS to above 25% whilst other OI's such as *Pneumocystis carinii* pneumonia and *Toxoplasma* encephalitis are decreasing (Hoover *et al.* 1993).

Table 1.2: Summary of CDC Classification of HIV Infection.

I **Acute Infection** (seroconversion)

II **Asymptomatic Infection**

III **Persistent Generalised Lymphadenopathy**

IVA **Constitutional disease**
one or more of: fever > 1 month, involuntary weight loss > 10% of baseline, diarrhoea > 1 month (in absence of causes other than HIV)

IVB **Neurological disease**
dementia, myelopathy, peripheral neuropathy (in absence of causes other than HIV)

IVC **Secondary infectious disease** (symptomatic or invasive)

IVC1 **Opportunist:**

Pneumocystis carinii pneumonia	Cryptococcosis
Chronic Cryptosporidiosis	Histoplasmosis
Toxoplasmosis	Mycobacterial infection with *M.Avium* intracellulare or *M.Kansasii*
Extra-Intestinal Strongyloidiasis	Cytomegalovirus Infection
Isosporiasis	Chronic Mucocutaneous HSV Infection
Herpes Simplex Ulcer > 1 month	Extra pulmonary Tuberculosis
Oesophageal or pulmonary candidiasis	Progressive multifocal Leukoencephalopathy

IVC2 **Other**

Recurrent Salmonella Bacteraemia	Nocardiasis
Pulmonary Tuberculosis	Multidermatomal Herpes Zoster

IVD **Secondary cancers**
Kaposi's Sarcoma: Non Hodgkin's Lymphoma (small non-cleaved lymphoma or Immunoblastic sarcoma); Primary Lymphoma of the brain

IVE **Other conditions**
eg: neoplasms other than those in D, or recurrent infections other than those in C.

1.15 HCMV Retinitis

Almost every organ system has at one time been reported to be affected by HCMV. Human CMV retinitis constitutes 85% of all HCMV disease in patients with AIDS, is the commonest ocular opportunistic infection and the leading cause of blindness in these patients. Indeed, it is now estimated that more than 20,000 new cases of HCMV retinitis occur annually in the United States (Jabs, 1996). Unilateral visual field loss or decreased visual acuity is the patients' most frequent presenting complaint and pain is classically absent. In 35% of cases bilateral disease is found at examination (Jabs, 1996). The diagnosis of HCMV retinitis is a clinical one as outlined by Holland in 1989 and can be mapped to one of three demarcated areas in the retina (Holland *et al.* 1989) as shown in Figure 1.2. The characteristic findings on ophthalmoscopy are patches of retinal whitening due to oedema and necrosis with variable amounts of retinal haemorrhage. The pattern of haemorrhage is usually one of perivascular sheathing. If left untreated, these lesions enlarge in a "brushfire" pattern (Holland and Shuler, 1992). These untreated lesions can double or treble their size over a one month period with a median progression rate of 24.0 μm/day (Holland and Shuler, 1992). Sixty percent of unilateral retinitis will progress to involve the fellow eye if left untreated. The term retinitis progression applies to progression of a lesion of HCMV retinitis by 750μm to allow the comparison of the efficacy of drugs in clinical trials (Anonymous. 1992). The suitability of this measurement in the routine clinical setting is now being questioned and more appropriate measures of treatment efficacy, such as visual outcome and quality of life, are being considered (Wu *et al.* 1996). Retinal detachments are common in HCMV retinitis and add significantly to the severe visual morbidity of the disease. The risk of retinal detachment increases with time and the one year cumulative probability of a detachment ranges from 50-61% (Anonymous. 1992).

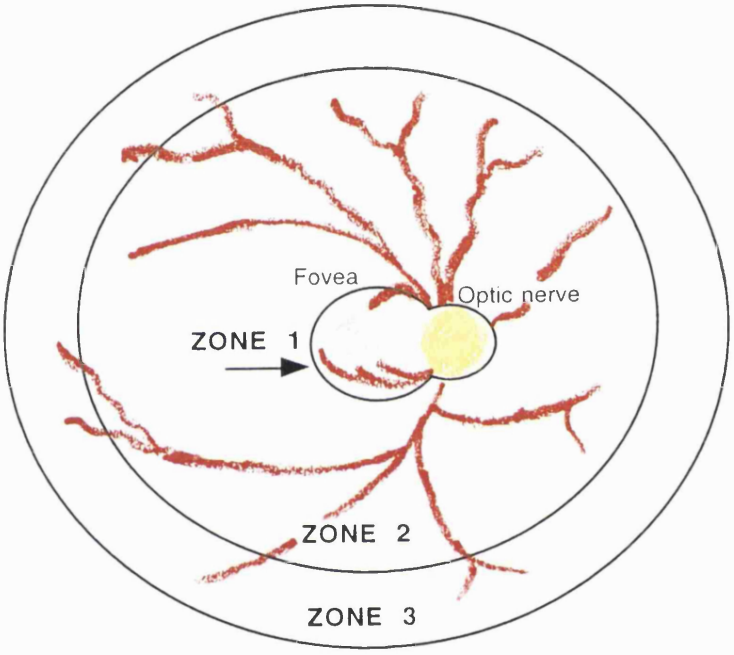


Figure 1.2: Diagram of the retina illustrating zones 1-to-3 used to identify the location of retinal lesions.

1.15.1 Pathogenesis of HCMV Retinitis

The pathogenesis of HCMV retinitis is still poorly understood. Early studies of post-mortem specimens found HCMV in all layers of the retina except the choroid and the endothelium of the retinal microvasculature (Pepose *et al.* 1985). These authors concluded that seeding of the retina was due to haematogenous spread through breaks in the capillary endothelium. More recent studies, in polarised human retinal pigment epithelial cells (RPE), have identified that the apical surface of the retinal pigment epithelial cells have receptors for HCMV. In these cells HCMV infection proceeds predominantly from the apical membrane and progeny virions can then spread from cell to cell via the lateral membranes independent of infection in the choroidal capillaries (Tugizov *et al.* 1996). This penetration of the apical membrane of polarised RPE cells was blocked by monoclonal antibodies to HCMV gB with little effect on HCMV spread across the lateral membranes. Further work from this group showed that the lateral cell to cell spread in these polarised RPE cells is facilitated by the accessory HCMV glycoprotein US9 (Maidji *et al.* 1996). HCMV US9 deletion mutants were unable to spread across the lateral membranes whilst apical binding to the cells was unaffected. With the establishment of 'animal models' for HCMV retinitis, as discussed in section 1.9, and the increasing availability of advanced molecular techniques such as *in situ* PCR and hybridisation to study post-mortem samples, the full pathogenesis of the mechanism of HCMV spread throughout the retina could be understood in the near future.

1.16 Other Systemic HCMV Disease

1.16.1 Gastrointestinal HCMV Disease

The gastrointestinal system is the second commonest organ infected by HCMV occurring in up to 15% of HIV infected patients (Jacobson and Mills, 1988). Clinical syndromes associated with HCMV infection include, in decreasing order of frequency, oesophagitis, colitis, gastritis, hepatitis, cholangitis and pancreatitis (Dieterich and Rahmin, 1991). In the gastrointestinal tract, infection usually manifests with inflammation and ulceration of the mucosa, pain and often haemorrhage. Patients may experience daily diarrhoea, often with bloody stools, and weight loss. Perforation is a rare occurrence but is responsible for up to 33% of all surgery required in patients with AIDS (Dieterich *et al.* 1995). Concomitant infection of the GI tract with other opportunist infections (OI) (such as *cryptosporidium, giardia*, and *microsporidium*) is common and symptoms often persist even after adequate treatment for the HCMV infection.

1.16.2 HCMV in the Central Nervous System

HCMV encephalitis often develops insidiously and diagnosis used to be made most frequently at post-mortem. Two clinically and neuropathologically distinguishable syndromes have been associated with HCMV encephalitis (McCutchan, 1995); (1) a widely distributed diffuse encephalitis involving the cortex, basal ganglia and cerebellum that is micronodular on pathological examination, and (2) a necrotising ventriculoencephalitis that results in necrosis of cranial nerves leading to cranial nerve deficits, delirium and rapid progression to death. Pathologically these two conditions may correspond to HCMV viraemia through the foot processes of the astrocytes and choroid plexus respectively (Griffiths and McLaughlin, 1997). Focal signs are unusual unless the lesion is in the brain

stem but seizures are common. Clinically HCMV encephalitis may be indistinguishable from HIV dementia, although survival in the latter is much longer due to the availability of effective treatment (Griffiths and McLaughlin, 1997). More cases of HCMV encephalitis are being seen in patients with HCMV retinitis who are living longer despite therapy with ganciclovir (Berman and Kim, 1994). Indeed, the relative risk of developing HCMV encephalitis after a previous diagnosis of HCMV retinitis is 9.5 and increases to 13 if the retinitis is present in retina adjacent to the optic nerve (Bylsma *et al.* 1995).

1.16.3 HCMV in the Peripheral Nervous System

A distinct syndrome of polyradiculopathy associated with HCMV infection is becoming increasingly common. Polyradiculopathy is due to HCMV infection in the spinal cord and can present as a medical emergency with acute spinal cord compression and a cauda equina lesion. A more insidious presentation may be with leg weakness and symmetrical sensory signs progressing to a flaccid paraparesis and bladder involvement (Behar *et al.* 1987). Both encephaltits and radiculitis carry a grave prognosis and response to monotherapy with either ganciclovir or foscarnet is poor (de Gans *et al.* 1990). Combination therapy with the two drugs may be a better option (Kim and Hollander, 1993), particularly with the emergence of HCMV strains in the CSF resistant to ganciclovir following long term treatment for HCMV retinitis (Smith *et al.* 1996).

1.16.4 HCMV and the Adrenal Gland

In many post-mortem studies on AIDS patients HCMV has been found to be highly tropic for the adrenal gland which is often the most frequently infected organ (Glasgow et al. 1985; Tapper *et al.* 1984). Symptoms of cortisol deficiency such as fatigue and dizziness

due to postural hypotension are common in late stage HIV infection and are therefore often misdiagnosed as sequelae of HIV infection. However, adrenal dysfunction due to HCMV infection is now being seen more frequently due to the heightened clinical awareness of a condition that was always considered to be a diagnosis made at post mortem. In a series of 74 post-mortems on AIDS patients, 37(50%) were found to have HCMV infection and in 84% of these the adrenal gland was involved (Pulakhandam and Dincsoy, 1990). Only 3 patients had ante-mortem adrenal function studies performed due to perceived symptoms of adrenal insufficiency but 30 patients were found retrospectively to have abnormal electrolyte (Na/K) values. More usual signs of adrenal insufficiency seen in the immunocompetent host, such as hyperpigmentation and profuse sweating, were absent. The most frequent finding, and probably the most useful clinical suggestion of a diagnosis of adrenal insufficiency, was postural hypotension.

1.16.5 HCMV Pneumonitis

In contrast to the high frequency of HCMV pneumonitis seen in the BMT patients, HCMV pneumonitis is an uncommon diagnosis in the HIV infected individual where cell mediated immunity is progressively and irretrievably depressed, consistent with the hypothesis of immunopathologically mediated disease. Two studies have found that HCMV detected in bronchoalveolar lavage (BAL) was neither a predictor of mortality nor associated with an increased prevalence of later HCMV disease and patients made a good recovery without anti-HCMV therapy (Millar *et al.* 1990; Bozzette *et al.* 1992). Two cases of proven HCMV pneumonitis in patients with HIV seroconversion illnesses have been reported. In both cases their CD4 counts were high (ie.CD4 >500 cells/μl) suggesting that they were able to mount a T cell response causing the immunopathology as documented in BMT patients

with pneumonitis (Squire *et al.* 1992).

1.17 Diagnosis of HCMV Prior Infection in Patients with HIV

As previously noted, prior HCMV infection in the HIV infected patient group is almost universal. At initial diagnosis of HIV infection all patients are routinely screened for evidence of prior infection with HCMV using an ELISA specific for HCMV IgG. This result is clinically very important as any patient found to be HCMV IgG negative must have their notes clearly marked for the use of HCMV negative blood and blood products. Serology for HCMV can be repeated annually to assess the patients management with regard to HCMV negative blood products and the suitability for trials of anti-herpesvirus therapies.

1.18 Diagnosis of Invasive HCMV Disease in the HIV Infected Patient

Where HCMV infection is thought to involve a particular organ system it is recommended that biopsies should be taken to aid the diagnosis. The characteristic findings of HCMV in histological biopsy sections are of intranuclear "owl's eye" inclusions which have a surrounding halo and marginated chromatin. The finding of these inclusions in sections of the gastrointestinal tract, liver and biliary tree, brain or skin is almost always indicative of invasive disease (Klatt and Shibata, 1988; Toome *et al.* 1991) and appropriate treatment should be instigated. One exception to this rule is the finding of HCMV in bronchoalveolar lavage samples (BAL) or transbronchial biopsy specimens. As discussed in section 1.16.5 the finding of HCMV in the BAL of a patient with AIDS does not confirm pneumonitis and any other pathogens found should be appropriately treated first and anti-HCMV therapy instigated only if the patient does not respond to the therapies aimed at

controlling the other opportunistic infections present.

1.18.1 HCMV Retinitis

HCMV retinitis is a clinical diagnosis. However, in difficult cases of atypical retinitis further specimens may be required to confirm the diagnosis and exclude other pathogens. The analysis of vitreous fluid by PCR has enabled the rapid differentiation of HCMV retinitis from other less common retinopathies associated with herpesvirus infections (Mitchell *et al.* 1994). The presence of detectable HCMV and VZV DNA was associated with disease whereas the presence of HSV-1, EBV and HHV6 was not. This has significant implications for the patients prognosis and treatment as VZV ocular infection is associated with an aggressive acute retinal necrosis that responds poorly to treatment, the drug of choice being high dose aciclovir (Margolis *et al.* 1991). The detection of herpesvirus DNA in ocular fluid has been shown to be more sensitive than the analysis of intraocularly produced antibodies for confirming a diagnosis of HCMV retinitis (Doornenbal *et al.* 1996).

1.18.2 HCMV Gastrointestinal Disease

A clinical diagnosis of HCMV ulceration in the upper gastrointestinal tract should be confirmed by histological examination of biopsies and by virological culture and DEAFF testing of biopsies and brushings. A positive result from these methods has been shown to direct the physician to the appropriate treatment and ultimately achieve a better clinical response from the patient (Dorigo-Zetsma *et al.* 1996). The polymerase chain reaction has been applied to DNA extracted from stool samples in AIDS patients with intestinal symptoms (Michel *et al.* 1995). HCMV PCR was positive in patients 8 of 36 patients, 4 of whom had histologically confirmed HCMV colitis. However it was also positive in one

patient with intestinal symptoms and 3 patients with systemic HCMV infection but no gastrointestinal symptoms. This procedure therefore needs to be evaluated further before being used as a clinical tool.

1.18.3 HCMV Related Neurological Disease

Diagnosis of HCMV infection of the central nervous system (CNS) had been mostly documented on post-mortem examinations of AIDS patients (Kure *et al.* 1991). Using conventional diagnostic techniques the diagnosis of HCMV infection in the CNS before death has been very difficult due to clinical ambiguity and the difficulty of obtaining biopsy specimens. Consequently treatment is often empirical and there have been no clinical trials to elucidate the most appropriate therapy and expected responses to such therapy. Two retrospective analyses of ante-mortem cerebrospinal fluid (CSF) found that there was a very strong correlation (92% and 88%; (Arribas *et al.* 1995; Achim *et al.* 1994), respectively) between the presence of HCMV DNA and evidence of HCMV CNS disease in sections of brains taken at post-mortem. The sensitivity and specificity of CSF examination for HCMV by PCR has been confirmed in prospective studies of AIDS patients with neurological disease (Gozlan *et al.* 1995) and (Fox *et al.* 1995). One study compared the quantity of HCMV DNA in the CSF with disease and found that HCMV CSF copy number was significantly greater in the 12 patients with HCMV polyradiculopathy than the 9 patients with HCMV encephalitis (mean DNA copies/µl CSF 11,982 vs 7,747 respectively) (Shinkai and Spector, 1995). Evaluation of 7 patients after treatment with ganciclovir for HCMV CNS disease found that 3 patients had become HCMV DNA negative in their CSF and 4 had lower levels of HCMV DNA than before treatment was started (Cinque *et al.* 1995). A similar reduction in HCMV DNA in CSF following the introduction of high dose ganciclovir

therapy has also been reported in two patients with HCMV polyradiculopathy and one with HCMV encephalitis (Shinkai and Spector, 1995). The decline in HCMV DNA copy number in CSF ranged from a four-fold decline at day 7 to a seventy-fold decline after three weeks of treatment. However, there was no correlation in either of these trials between reduction of HCMV DNA in the CSF and clinical outcome so the use of this assay in the clinical management of patients remains to be defined.

1.19 Detection of Systemic HCMV Infection

1.19.1 Cell culture

Cell culture has been regarded as the gold standard for the detection of HCMV in blood or urine samples and remains the standard against which all new techniques must be compared. Clinical specimens are inoculated either onto permissive human fibroblasts (either from embryo lungs or foreskins) or MRC-5 cells (as they are the only cell types which reproducibly support HCMV replication *in vitro*). However, one major disadvantage of cell culture is that the appearance of typical CPE takes at least 7-16 days and the cultures have to be maintained for 21 days before being reported as negative. Toxicity of some samples in culture can also be a problem and may lead to a mis-diagnosis. As most samples are taken from severely immunocompromised patients this is an unacceptedly long and dangerous time to wait for a result before commencing treatment.

1.19.2 Rapid Culture Systems

In order to accelerate this diagnostic process, assays that detect expression of immediate early and early proteins of HCMV in cell culture 24 hours after inoculation are now commonly used. In the UK this assay uses immediate early monoclonal antibodies

bound followed by fluorescein conjugated anti-mouse antibody to detect HCMV protein and is therefore called the DEAFF test (for Detection of Early Antigen Fluorescent Foci; (Griffiths *et al.* 1984)). A comparison of this technique with conventional cell culture has shown the sensitivity of DEAFF to be 78% vs 76% for the former (Pillay *et al.* 1992). In the US the shell vial assay (after the name of the container) is the most commonly used assay for the rapid detection of HCMV and also uses monoclonal antibodies against immediate early antigens (Gleaves *et al.* 1984). The sensitivity of the test is between 70 to 90% with a specificity of almost 100%.

1.19.3 Antigenaemia

Monoclonal antibodies can also be used to detect HCMV antigens directly in polymorphonuclear leukocytes (PMNLs). The most frequently used targets for this assay are the antibody reactive polypeptides of UL83 (pp65). Protein pp65 is the lower matrix protein and is the major antigen detected in circulating leucocytes (Gerna *et al.* 1991). Results from this assay can be obtained in 5 hours and the sensitivity of the technique is at least equivalent to other rapid culture methods and conventional cell culture (van-der-Bij *et al.* 1988). Moreover, antigen stained cells can be counted relative to the known input of PMNLs allowing for a semi-quantitative (if subjective) assay of HCMV viraemia. Particular advantages of this assay are that special facilities are not required and the reagents for the assay are cheaper than for PCR but the demands on staff time are higher. Recently a comparison between a commercial HCMV antigenaemia assay (the 'Brite' kit) and a HCMV reference antigen assay found the kit to be as sensitive and specific as the reference method with the advantage of fixed positive controls (Landry *et al.* 1996). The availability of a standard antigenaemia assay that could be used and compared across different clinical

laboratories would be a distinct advantage. Disadvantages include the need to collect and process the sample rapidly (so stored samples cannot be analysed) and quantification is labour intensive as the foci from $>10^5$ cells need to be counted (Landry and Ferguson, 1993). One concern regarding antigenaemia, however, is the uncertainty of the source of pp65 within the PMNLs. The pp65 protein may be phagocytosed, virion derived protein that could be a carry over from a previous infection and does not necessarily equate with an acute infection. Antigenaemia has been shown to be indicative of HCMV infection in prospective studies of immunocompromised groups such as renal, thoracic and liver transplant recipients (van-der-Bij *et al.* 1988; Schmidt *et al.* 1996; Barber *et al.* 1996).

The use of antigenaemia in predicting disease in HIV infected individuals has not been as extensively studied. In one study of 122 patients with CD4 counts <100 cells/μl pp65 antigenaemia was found to have a good negative predictive value (NPV) at 95% for HCMV disease but a positive predictive value (PPV) of only 47% and a sensitivity of 91% (Bek *et al.* 1996). If a positive result was taken to be > 5 positive cells per slide the PPV could be increased to 63% but the sensitivity and NPV decreased reciprocally (67% and 84% respectively). Reynes *et al* also found that the PPV of pp65 antigenaemia could be increased from 45% to 93% if a positive result was taken as >100 cells per slide, but the NPV was reduced from 94% to 80% (Reynes *et al.* 1996). This suggests that the number of positive cells per 2 x 10^5 PMNLs taken as a positive result is crucial to the sensitivity and specificity of the assay and should be considered when interpreting results. In a prospective study of 144 HIV positive patients presenting with symptoms of a febrile illness the sensitivity and specificity for HCMV disease and a positive pp65 antigenaemia assay was 86% (Salzberger *et al.* 1996). Only three of fifteen patients who were pp65 positive with no evidence of disease at that time went on to develop HCMV disease after 6-9 months follow-up. In 10

patients followed up for more than 6 months in another study there was a mean time delay of 102 days (range 40-172) between first positive antigen result and subsequent HCMV disease, suggesting that this assay may have some prognostic value for screening patients at high risk of HCMV disease (Reynes *et al.* 1996).

1.19.4 DNA Detection

Initial studies in the early 1980's detected HCMV nucleic acids in clinical specimens using hybridisation assays. Although in situ hybridisation has been shown to detect HCMV DNA in tissue specimens in cells that do not appear to be infected by histological criteria (Myerson *et al.* 1984), the sensitivity of these assays was significantly less than conventional cell culture (Pillay and Griffiths, 1992).

These methods have now been superseded by the polymerase chain reaction (PCR). This method utilises specific primers complementary to DNA sequences present in the target HCMV DNA of interest. This target sequence is subsequently amplified in repeated cycles of denaturation, elongation and annealing in the presence of a thermostable DNA polymerase (Saiki *et al.* 1988). There have now been many reports proving the higher sensitivity of PCR for the detection of HCMV in clinical specimens in many immunosuppressed patient groups as well as congenitally infected children (Einsele *et al.* 1991; Ratnamohan *et al.* 1992; Demmler *et al.* 1988; Kidd *et al.* 1993). Other advantages of PCR include the short period of time to achieve a result, the ability to analyse multiple different samples in one analysis (as samples can be stored prior to use) and the high reproducibility of the assay. The disadvantages include the cost of the reagents and the possibility of contamination due to the high sensitivity of the assay. This, however, can be minimised following a strict protocol including the use of three different rooms for each

stage of the process (Kwok and Higuchi, 1989), or the inclusion of dUTP in the amplification followed by the pre-treatment of reagents with uracil-N-glycosylase.

HCMV DNA can be amplified from whole blood, leukocytes or plasma. It can also be amplified from urine, BAL, vitreous fluid and CSF. The target for HCMV DNA detection varies according to the particular preference of each laboratory as there have not been any formal head to head comparisons of each method for the qualitative detection of HCMV DNA. Whole blood has the advantage of amplifying HCMV DNA from all cells present as well as cell free virus in plasma. The use of a finite number of polymorphonuclear leucocytes has the advantage in neutropenic patients of controlling for changes in white cell counts with different therapies. A direct comparison of PCR from plasma, buffy coat and antigenaemia in liver transplant recipients found a 97.2% concordance between results from plasma and buffy coat and 94.3% concordance between both PCR methods and antigenaemia (Schmidt *et al.* 1996). Discordance was seen mostly in the early phase of HCMV infection where both PCR assays became positive up to three weeks before the antigenaemia assay. Detection of HCMV DNA in plasma in AIDS patients has been shown by Spector *et al* to correlate positively with peripheral blood leukocyte (PBL) cultures and the presence of visceral HCMV disease (Spector *et al.* 1992). Eighty three % of patients with HCMV retinitis were plasma PCR positive at diagnosis of retinitis compared to 78% for buffy coat cultures. Plasma PCR was also more likely to become positive with reactivation of retinitis after 6 months therapy. There was little evidence to suggest that infectious virus was present in plasma as only 2 of the 11 cultured plasma samples yielded infectious virus. Therefore plasma and whole blood PCR may be amplifying many non-infectious particles which may have implications when interpreting such results. A

comparative study of three PCR assays in thoracic transplant recipients found that primers amplifying a 149 bp region of gB were more sensitive than either the IE1 or pp150 primer sets at predicting HCMV disease (Barber *et al.* 1996). These gB primers are those utilised in the studies described later in this thesis.

1.20 Quantification of HCMV DNA in AIDS Patients

In order to provide more accurate prognostic information and insight into HCMV pathogenesis, the amount of HCMV DNA has been quantified in clinical specimens using a variety of different molecular techniques. Quantification of HCMV using both conventional cell culture methodology (Stagno *et al.* 1975) and molecular methods has been shown to have prognostic value in both renal transplant recipients (in urine) and (in blood) in bone marrow allograft recipients (Fox *et al.* 1992; Fox *et al.* 1995; Saltzman *et al.* 1992).

Probably the most accurate method of quantifying DNA is to co-amplify control in each reaction thus allowing for intra-reaction variation. This co-amplification has been applied to the IE1 gene region in the presence of an internal plasmid control and external standard. Gerna *et al* found that HCMV DNA levels in leukocytes are generally higher than those in plasma, persist for longer and appear to have a greater correlation with disease in AIDS patients (Gerna *et al.* 1994b; Zipeto *et al.* 1993). The PCR products were visualised using slot blots which were analysed and quantified by densitometric analysis. Co-amplification of the IE1 region and an internal control plasmid was also used by Zipeto *et al* to evaluate the relationship between HCMV DNA in the leukocytes and plasma of AIDS patients (Zipeto *et al.* 1995). Quantification of HCMV DNA in leukocytes was more sensitive and more accurate when compared to HCMV DNA in plasma although using both assays, levels

of HCMV DNA were significantly higher in the patients with HCMV disease compared to patients with asymptomatic HCMV infection. Rasmussen *et al* used a single round PCR to amplify HCMV DNA from buffy coat cells (BCC) and mononuclear cells (MNC) for semi-quantification using a colorimetric detection assay (Rasmussen *et al.* 1995). There was no evidence for selective infection of either MNCs or BCCs by HCMV. HCMV DNA was detected in 36% HIV positive patients with CD4 counts >100 cells/μl but at lower levels than the 64% patients with CD4 counts <100 cells/μl with no evidence of HCMV disease. The highest copy numbers (up to 5.5 $\log_{10}$ copies/μg DNA) were seen in the patients with active HCMV retinitis. There was however a large variation in copy numbers between the three groups that questions the significance of the increasing viral load with symptomatic disease. Of interest, the authors noted that 3 out of the 10 patients with >100 copies HCMV DNA in BCC developed retinitis within 4 months. The quantification of HCMV DNA in the CSF has been discussed in section 1.18.3.

1.21 Value of HCMV Detection in Predicting Disease

Unlike transplant recipients who have a defined risk period for disease, the AIDS patient is at risk of developing HCMV disease once their CD4 count falls to below 100 cells/μl. This makes the cost/benefit ratio for the prophylactic treatment of all patients at risk of HCMV disease very high. Therefore, certain parameters are necessary to identify those patients most at risk of subsequent disease. Two studies have analysed conventional cell culture for HCMV in blood and urine as a predictor of invasive HCMV disease in AIDS patients. Zurlo *et al* found that there was a poor correlation between HCMV viraemia/viruria and the development of HCMV disease with a PPV of 35% and 28% respectively and suggested that the presence of HCMV viraemia/viruria correlated only with

a poor immune response rather than current or future HCMV disease (Zurlo *et al.* 1993). This compares to the PPV of 50% quoted by Salmon *et al* for patients developing HCMV organ involvement 16.6 ± 7 months if HCMV was cultured from blood at the time of their AIDS diagnosis (Salmon *et al.* 1990). Therefore, over a 16 month follow-up of 71 AIDS patients, 50% of those with HCMV viraemia developed HCMV end organ disease compared to 9% of non-viraemic patients. More recently, Gellrich *et al* found that HIV positive patients, with a CD4 count < 50 cells/μl, who were HCMV viruric (by shell vial cultures) were seven times more likely to develop HCMV retinitis than those who were not viruric (Gellrich *et al.* 1996).

Recent studies have concentrated on the use of qualitative PCR as a more rapid and sensitive means of predicting HCMV disease. Hansen *et al* used a nested PCR on previously stored serum in a prospective study of patients with HCMV retinitis (Hansen *et al.* 1994). Both the PPV (85%) and the sensitivity and specificity of the PCR (74% and 88% respectively) to detect patients with disease were much higher than those for cell culture (Zurlo *et al.* 1993; Salmon *et al.* 1990). However, the true predictive nature of the study by Hansen *et al* is difficult to define as many of the patients had HCMV disease at the time the PCR was performed, ie it was partly composed of cross-sectional data. Further prospective studies using quantitative PCR are needed to establish if there is a threshold level of HCMV DNAemia above which asymptomatic patients will develop HCMV disease.

1.22 Antiviral Agents to Control HCMV Disease

To date only two drugs; ganciclovir and foscarnet are approved for use in life threatening HCMV disease in the United Kingdom. There are however, many anti-viral

agents currently in phase I-III clinical trials.

1.22.1 Ganciclovir

Ganciclovir (GCV) is the antiviral agent used as first line treatment for HCMV infection. GCV is a analogue of the purine nucleoside guanosine. GCV is acyclic as defined by the absence of the 2' carbon atom of the deoxyribose moiety. GCV does however have the 5' and 3' hydroxyl moieties which are required for chain elongation. GCV inhibits viral DNA in two ways; firstly GCV triphosphate competes with the natural guanosine triphosphate for binding to the elongating DNA chain in the presence of DNA polymerase and is thus incorporated into the DNA as shown in Figure 1.3. Secondly, GCV terminated DNA is a poor substrate for chain elongation and therefore growing DNA chains terminate prematurely (Marshalko *et al.* 1995). Once taken into cells GCV is monophosphorylated by the virally induced gene UL97 and then triphosphorylated into its active form by cellular enzymes that can be incorporated into the viral DNA (Sullivan *et al.* 1992). As GCV closely resembles the natural nucleoside dG there is also the possibility of cellular toxicity. The effective inhibitory concentration (IC_{50}) of GCV for HCMV is 0.3 - 10 μM whilst bone marrow colony forming cells are inhibited at an IC_{50} of 39 μM resulting in a narrow therapeutic window. This partly explains why the commonest side effects associated with GCV treatment are neutropenia, anaemia and thrombocytopenia. These conditions can now be controlled by the concurrent use of granulocyte colony stimulating factor (GCSF) and recombinant erythropoeitin. GCV was originally only administered intravenously; however, more recently an oral formulation has been licensed but is hampered by a very low oral bioavailability. In addition, a slow release intraocular implant is available and a valine ester pro-drug of GCV is undergoing clinical trials.

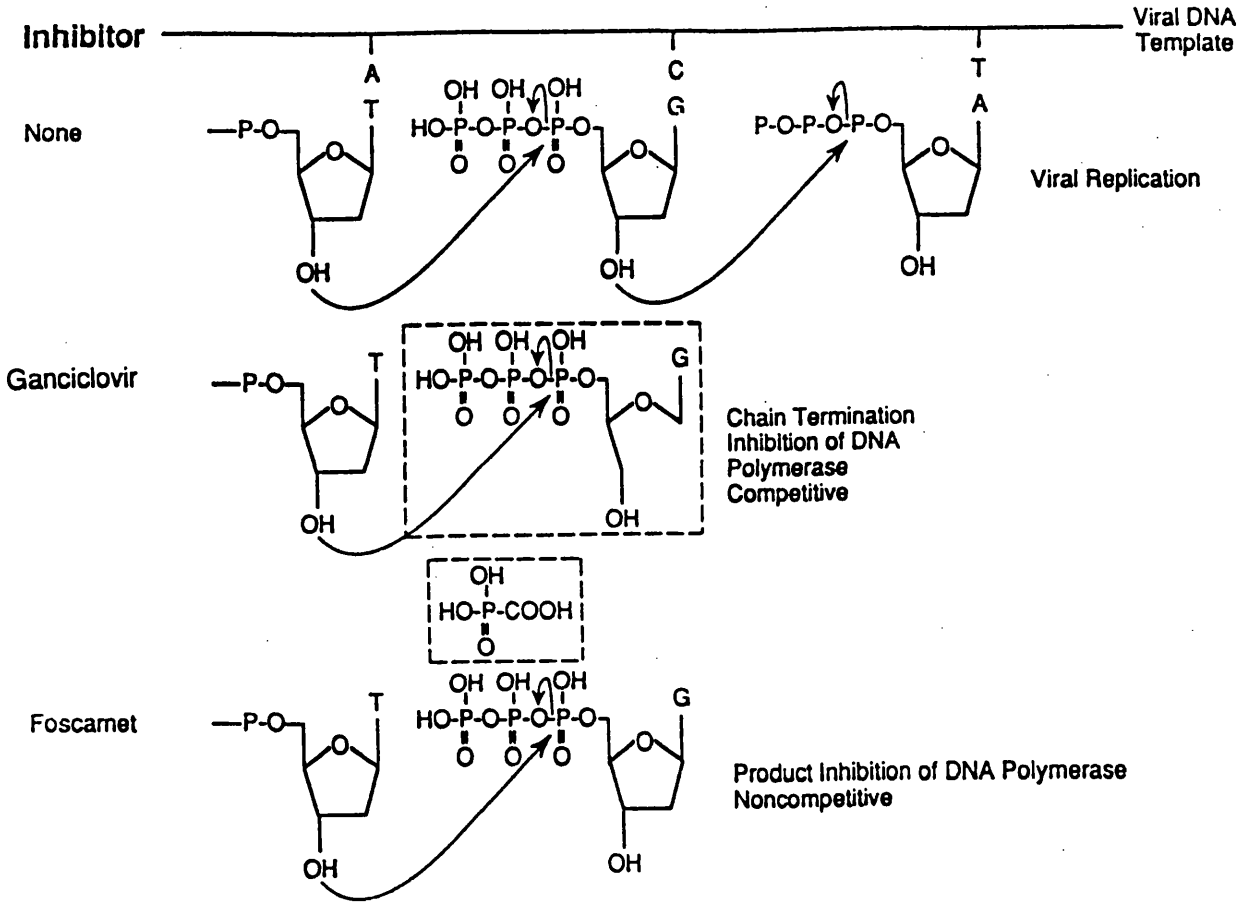


Figure 1.3: Sites of action of ganciclovir triphosphate and foscarnet on HCMV replication. (Re-drawn from "Clinical Aspects of Treatment of Cytomegalovirus"; Pillay *et al*, in Antiviral Chemotherapy Ed Jeffries D J & de Clereq E, Wiley 1995).

1.22.2 Foscarnet

Foscarnet (PFA) is a pyrophosphate analogue that binds to the HCMV DNA polymerase and reversibly inhibits elongation of the viral DNA chain by product inhibition, (see Figure 3.1). PFA is also a non-competitive reversible inhibitor of HIV reverse transcriptase (Wagstaff and Bryson, 1994). Unlike GCV, PFA does not need to be phosphorylated to be activated. Foscarnet is almost exclusively renally excreted unchanged and nephrotoxicity related to tubulointerstitial lesions is a very common side effect (Wagstaff and Bryson, 1994). Hydration with normal saline prior to each PFA infusion reduces the severity of renal dysfunction in most patients. PFA is poorly absorbed orally and must therefore be given intravenously or intravitreally although a liposomal encapsulated form of PFA is being studied and seems to offer some protection against the renal toxicity associated with standard PFA (Omar *et al.* 1995).

1.22.3 Cidofovir

Cidofovir (1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC)) is a nucleotide analogue of 2' deoxycytidine monophosphate which, when activated to its diphosphate form, competitively inhibits HCMV DNA polymerase. Cidofovir (CDV) has been recently licensed by the Food and Drug Administration in the United States for the treatment of HCMV retinitis but has yet to be licensed in the United Kingdom and is currently only available through expanded access programmes. CDV is a much more potent *in vitro* inhibitor of HCMV than either GCV or PFA and has a much longer half-life allowing for fortnightly dosing regimens (De Clercq, 1993). Similar to PFA, nephrotoxicity is a significant problem with CDV necessitating intravenous hydration and concomitant oral probenecid to decrease renal clearance of the drug and prevent deposition of CDV in the

renal tubules.

1.22.4 Other Investigational Anti-viral Agents with Activity Against HCMV

There are currently many agents in phase I/II and III development. These include the phosphonate derivative adefovir (currently being studied as the oral pro-drug bis-pom-PMEA) which has a very broad anti-viral spectrum including hepatitis B virus and HIV itself. Lobucavir (carbocyclic oxetanocin G) is another compound with a much broader anti-viral spectrum and with fair oral bioavailability and tolerability in phase II studies. There are exciting developments in new classes of antiviral agents such as the anti-sense oligonucleotide ISIS 2922 which is given weekly by intravitreal injection. ISIS 2922 is a 21-mer oligonucleotide complementary to the IE2 gene that is stabilised against digestion by normal cellular enzymes by the inclusion of phosphorothioate moieties. Benzidimazole ribosides such as BDCRB are drugs targeted at the UL89 terminase complex. However, due to poor *in vivo* results from a high degree of protein binding, the lead compound now in development from that group is another BDCRB derivative, BW 1263, that inhibits HCMV DNA synthesis. BW 1263 does not interfere with the UL89 terminase complex but its exact mechanism of action has yet to be elucidated. MSL-109, a monoclonal antibody against gH, has been shown in phase II studies to be ineffective and current studies have been stopped.

1.23 Treatment Strategies for HCMV Infection/Disease

Treatment strategies for HCMV infection have mainly been conceived and studied in the transplant setting where patients have a much more clearly defined risk of HCMV infection and are at highest risk for a finite period of time. Strategies that have proven to be very effective in the transplant recipient aim to reduce HCMV infection before disease ensues. Prophylaxis is given to high risk patients in the peri-transplant period before HCMV infection is detected. At this stage, the risk of HCMV disease to the patient is low so the acceptable toxicity of the drugs used must also be low. Aciclovir has fulfilled these criteria in both bone marrow transplant recipients and renal transplant recipients at risk of primary HCMV infection (Prentice *et al.* 1994; Balfour *et al.* 1989). GCV prophylaxis in BMT recipients reduces HCMV infection and disease but has no benefit on survival due to the increased incidence of neutropenia and subsequent bacterial sepsis (Goodrich *et al.* 1993; Winston *et al.* 1993). In liver transplant recipients low dose GCV prophylaxis significantly reduces HCMV infection and disease in both HCMV antibody positive and negative recipients compared to aciclovir (Winston *et al.* 1995).

Patients who have evidence of systemic HCMV replication either by molecular methods or standard culture methods have a much higher risk of HCMV disease. Therefore the greater toxicity of an antiviral compound would be acceptable. This form of "pre-emptive" therapy (Rubin, 1991)has been successfully used in BMT patients where GCV given after systemic detection of HCMV significantly reduced both HCMV disease and mortality (Goodrich *et al.* 1991; Schmidt *et al.* 1991).

Obviously once HCMV has become established there is a significant morbidity and a high probability of mortality and high dose regimens of intravenous therapy are warranted to control the disease. To date this has been the mainstay of management of HCMV disease

in the HIV infected patient although more trials investigating prophylaxis and preemptive therapy are underway.

1.24 Treatment of Established HCMV Disease in the HIV Infected Individual

1.24.1 Induction Therapy

The standard protocol for treating newly diagnosed HCMV retinitis is a 14-21 day period of high dose (ganciclovir 5 mg/kg/bd or foscarnet 90 mg/kg/bd) intravenous (iv) induction therapy followed by life long maintenance therapy. Two studies have investigated the rationale for immediate vs deferred therapy in patients with non-sight threatening peripheral retinitis. For patients randomised to receive 14 days ganciclovir induction therapy then once daily maintenance, time to progression for the immediate treatment group was 50 days compared to a significantly shorter 14 days for the deferred group (Spector *et al.* 1993). Similar results were seen with foscarnet where patients receiving immediate treatment (21 days of foscarnet induction therapy) had a significantly longer time to progression than those randomised to deferred therapy (84 vs 21 days) (Palestine *et al.* 1991). There have been no controlled trials to compare the efficacy of 14 vs 21 days of either of the above agents for induction therapy. Over 80% of patients will have a significant clinical response to the drug after 14 days as evidenced by lack of progression of the retinitis as well as a decrease in the border of opacification (Anonymous. 1992; Holland *et al.* 1989). A head to head comparison of ganciclovir vs foscarnet induction therapy for 21 days showed that 86% patients receiving ganciclovir had a complete response at three weeks compared to 68% on foscarnet (Moyle *et al.* 1992). Virological response (in the presence of a complete clinical response) to induction therapy was seen in 83% of patients receiving ganciclovir (Buhles *et al.* 1988)and 73% in those receiving foscarnet induction therapy (Gerna *et al.* 1994a). However, the

relationship between the continued presence of HCMV in the blood after induction therapy and the subsequent clinical course of the patient has not yet been fully elucidated.

HCMV disease in the gastrointestinal tract and the central/peripheral nervous system is treated with high dose induction therapy with either ganciclovir of foscarnet for at least 21 days and up to six weeks depending on the clinical and histological response achieved.

1.24.2 Maintenance Therapy

A large randomized unblinded study comparing ganciclovir 5mg/kg/d vs foscarnet 90mg/kg/d found the two agents to be equally efficacious in the secondary prophylaxis of HCMV retinitis, with median times to first progression of 56 and 59 days respectively (Anonymous. 1992). Interestingly, there was a survival advantage for foscarnet with a median survival of 12.6 months for patients in this group compared to 8.5 months for those treated with ganciclovir, even though there were more toxicity problems with foscarnet. The survival differences could not be explained by differences in antiretroviral therapies between the two groups although patients on ganciclovir received less AZT due to ganciclovir related neutropenia. Further studies have not found a survival benefit in favour of foscarnet and both ganciclovir and foscarnet have been shown to significantly decrease immune complex dissociated p24 antigenaemia (Anonymous. 1995a). The length of time to progression with maintenance foscarnet can be significantly increased using 120mg/kg instead of 90 mg/kg although this is associated with a commensurately large increase in toxicity (Holland *et al.* 1995).

In order to reduce both the inconvenience to the patient and the risk of infection with an indwelling catheter, oral ganciclovir 1000 mg tds has been compared with 5mg/kg iv ganciclovir od in patients with stable retinitis following a full induction course with ganciclovir (Anonymous. 1995b; Drew *et al.* 1995). Patients were assessed monthly both clinically and using masked retinal photographs assessed in a central reading centre. The times to progression are summarised in Table 1.3.

Although the time to progression of retinitis was not significantly shorter with oral ganciclovir when using masked fundoscopy there was a significant difference according to the clinician (who was aware of the patients' treatment arm). Retinal photographs are commonly used for the real time assessment of patients in the routine clinical setting and patients should be made aware of this potential time difference. Despite these differences in time to progression there were no significant differences in deterioration of functional vision or the development of new lesions in either study between the two arms. Further studies comparing 3g vs 4.5g vs 6g of oral ganciclovir with iv ganciclovir have shown a trend towards an increased time to progression with higher doses of ganciclovir (along with an increased incidence of drug associated neutropenia), but this was not significant and higher doses of oral ganciclovir have not been licensed (Lalezari *et al.* 1996). It is recommended that oral ganciclovir should be reserved for patients with zone 2/3 disease where the risk of progression and central spread of the retinitis is balanced with the benefit of taking oral therapy and not using daily iv infusions.

Table 1.3: Days to first progression of HCMV retinitis in two randomised controlled trials of oral vs intravenous ganciclovir.

	Days to Progression by Open Clinical Assessment		**Days to Progression by Masked Fundus Photographs**	
Trial/Study	**iv**	**oral**	**iv**	**oral**
European/Aust (AIDS 1995)	109	86	62	51
USA (NEJM 1995)	96	86	62	57

A more recent trial by the SOCA study group randomised patients with persistently active or relapsing retinitis, after reinduction, to either monotherapy with foscarnet 120mg/kg od or ganciclovir 10 mg/kg od or combination therapy with foscarnet 90 mg/kg od and ganciclovir 5 mg/kg od (Anonymous. 1996). Time to retinitis progression was significantly longer in the combination arm (4.3 months vs 2 months for ganciclovir and 1.3 months for foscarnet). There was also a survival benefit for the combination arm even though there were more drug related side effects. However this regimen entails at least 4 hours of drug infusion time each day contributing to a decreased quality of life with no significant improvement in visual acuity. For this reason new trials of anti-viral agents for HCMV retinitis include the measurement of quality of life along with the outcome of visual function as an integral part of the trial (Wu *et al.* 1996).

Maintenance therapy has been shown to offer no benefit in either time to recurrence or mortality in patients with gastrointestinal HCMV disease and is therefore not used routinely (Blanshard *et al.* 1995). High dose intravenous ganciclovir and foscarnet maintenance therapy has been used successfully in patients with HCMV polyradiculopathy although the prognosis remains poor.

1.24.3 Topical Therapy

Intravitreal therapy has the benefit of local treatment and high vitreous drug levels without the systemic side effects of ganciclovir or foscarnet. However, the disadvantages are the high incidence of systemic HCMV disease and infection in the fellow eye. Intravitreal injections of ganciclovir and foscarnet have been used since 1987. Ganciclovir is most commonly used at doses of 200µg/wk and foscarnet at 2000µg/wk as maintenance therapy

although there have been no controlled trials of intravitreal therapy (Diaz-Llopis *et al.* 1992; Engstrom and Holland, 1995). There is a slight increase in the incidence of retinal detachment and endophthalmitis compared with systemic treatment.

In order to lengthen the dosing schedule of intravitreal ganciclovir therapy a sustained release device has been developed that can be implanted into the eye and can give up to four months continuous GCV delivery into the eye. Median time to progression with the implant (which was changed electively every 4 months) was 226 days vs 56 days for standard iv ganciclovir treatment. Surgical complications are retinal detachment (11-30%), endophthalmitis (10%), and a transient reduction in visual acuity that recovers by day 14 post procedure and is reduced with increasing experience of the surgeon involved (Martin DF. *et al.* 1994). The risk of developing disease in the fellow eye is 50% at 6 months and 31% of patients developed biopsy proven CMV visceral disease (Martin DF. *et al.* 1994). Ongoing trials in the United States are using oral GCV to reduce the probability of systemic infection/disease in patients treated with the implant vs standard iv therapy. However, it is clear that further studies are needed to define those patients most at risk of systemic disease who could then receive appropriate therapy.

1.25 Primary Prophylaxis Against HCMV in HIV Infected Patients

Two randomised double-blind placebo controlled trials of oral ganciclovir 1g tds vs placebo have been completed in HIV positive patients with CD4 counts <100 cells/μl. Patients with a history of previous HCMV disease or exposure to anti-HCMV therapy were excluded. The study endpoints were time to HCMV disease or death. In the first study (1654), where the median CD4 count was 20 cells/μl and all patients had baseline indirect ophthalmoscopy, oral ganciclovir significantly reduced both the incidence of HCMV disease

(20% vs 39%) and HCMV retinitis (18% vs 39%) compared to placebo (Spector *et al.* 1996). All patients had baseline plasma samples taken for HCMV PCR and in preliminary results those patients who were HCMV PCR negative (ie received true prophylaxis) received the greatest benefit from oral ganciclovir. Patients who were HCMV PCR positive and received ganciclovir as pre-emptive therapy still received some benefit from the drug and a reduction in the incidence of HCMV retinitis if their baseline viral load was low. These results contrast markedly to a second study (CPCRA 023) where there was no significant difference in HCMV disease between oral ganciclovir and placebo (Brosgart *et al.* 1995). Although the median CD4 count in the CPCRA study was slightly higher and there was no baseline ophthalmoscopy, subset analyses of patients in the same groups as 1654 still failed to show any benefit for oral ganciclovir.

A much larger study of over 1227 patients was carried out by the AIDS Clinical Trials Group (ACTG) to study the efficacy of valaciclovir (the valine ester prodrug of aciclovir) vs high and low dose aciclovir at preventing HCMV end-organ disease (ACTG 204). There was a significant reduction in both HCMV disease (17.5% vs 11.7%) and HCMV retinitis (13.5% vs 9.8%) in the valaciclovir group (Feinberg *et al* 1996). In both studies there was a significant increase in side effects in the treatment arms. In the ganciclovir studies the use of GCSF for neutropenia and erythropoetin for anaemia was greatly increased for those patients receiving active drug rather than placebo, whereas the patients taking valaciclovir reported significantly more gastrointestinal side effects than those taking aciclovir. There was also an increase in mortality in patients receiving valaciclovir that led the DSMB to stop the trial prematurely. In the final analysis this trend persisted although it was not significant and its exact cause has not yet been explained. These agents are not without their side effects and,

unlike transplant recipients, where the risk of HCMV infection is a transient phenomenon, the HIV positive patient will require drug therapy for life. This has major cost implications as the cost/benefit ratio for oral ganciclovir is very high as to prevent one case of HCMV retinitis with oral ganciclovir 8.3 patients would have to be treated (Spector *et al.* 1996). In the US as each patients treatment costs $27,000, the cost of preventing one case of HCMV retinitis would be more than $223,000 (Nicklin, 1996).

From these early trials both oral ganciclovir and valaciclovir do certainly appear to be effective as prophylactic and pre-emptive therapeutic agents respectively. However, to improve the cost/benefit ratio it will be more reasonable to target these drugs, and hopefully more potent drugs that are in development, to the patients at highest risk of HCMV disease (as in the transplant setting).

1.26 The Significance of HCMV Resistance to Antiviral Agents

A laboratory derived HCMV ganciclovir resistant mutant was first identified in 1986 (Biron *et al.* 1985). Three years later Erice *et al* correlated the presence of HCMV resistance to ganciclovir in blood isolates with the poor clinical outcome of three immunocompromised patients (two with AIDS and one with chronic lymphocytic leukaemia) (Erice *et al.* 1989). As the usage of ganciclovir increased so did the identification of HCMV ganciclovir resistant isolates. Drew *et al* estimated the incidence of GCV resistance in AIDS patients receiving continuous maintenance therapy to be 7.6% (Drew *et al.* 1991). The isolates in this study came from urine and there was no correlation with the clinical outcome of the patient. On the basis of this study and other cumulative data, Drew *et al* proposed an IC_{50} of 12μM as the approximate cut off for HCMV GCV susceptibility studies (Drew *et al.* 1991). More recently however, an IC_{50} of 12μM has been equated with severe resistance and a lower IC_{50} of 6μM taken as the threshold for a diagnosis of GCV resistant HCMV particularly in the context of clinical trials. In 1992/3 the genotypic basis of HCMV resistance to GCV was established with the identification of the UL97 open reading frame and point mutations in the region V of DNA *pol* UL54 (Sullivan *et al.* 1992; Littler *et al.* 1992; Sullivan *et al.* 1993). However, the full extent of the correlation between clinical progression of retinitis and the appearance of resistant strains has yet to be defined. Certainly the majority of first progressions of HCMV retinitis appear to be due to treatment failure related to either sub-therapeutic intravitreal concentrations of drug (Arevalo *et al.* 1995) or poor patient compliance rather than the emergence of resistant viral strains. A prospective study of 49 patients with HCMV retinitis found no evidence of GCV resistance in pre-therapy blood isolates but overall 4% of patients had either a blood or urine culture of HCMV that was resistant to foscarnet, but this was not correlated with the subsequent clinical course of the patient (Jabs *et al.* 1996). Most patients

however, have no evidence of HCMV in blood or urine at the time of first retinitis progression and retinitis progression appears to be a local reactivation (Drew *et al.* 1991). HCMV strains showing *in vitro* resistance to GCV and foscarnet have been isolated from the blood and urine of patients with "clinically resistant retinitis" who had suffered multiple progressions of retinitis on treatment (Flores-Aguilar *et al.* 1993; Dunn *et al.* 1995). Two of the patients in the former study (Flores-Aguilar *et al.* 1993) had retinal biopsies (performed during the repair of a retinal detachment) that were reported as being HCMV PCR positive but the *in vitro* susceptibility of these specimens to anti-viral therapy was not reported (Flores-Aguilar *et al.* 1993). To date all of the pheno- and genotypic characterisation of HCMV resistant strains has been performed on virus from peripheral sites rather than the site of retinitis progression.

1.27 The Molecular Basis of Resistance of HCMV to Ganciclovir and Other Antiviral Agents

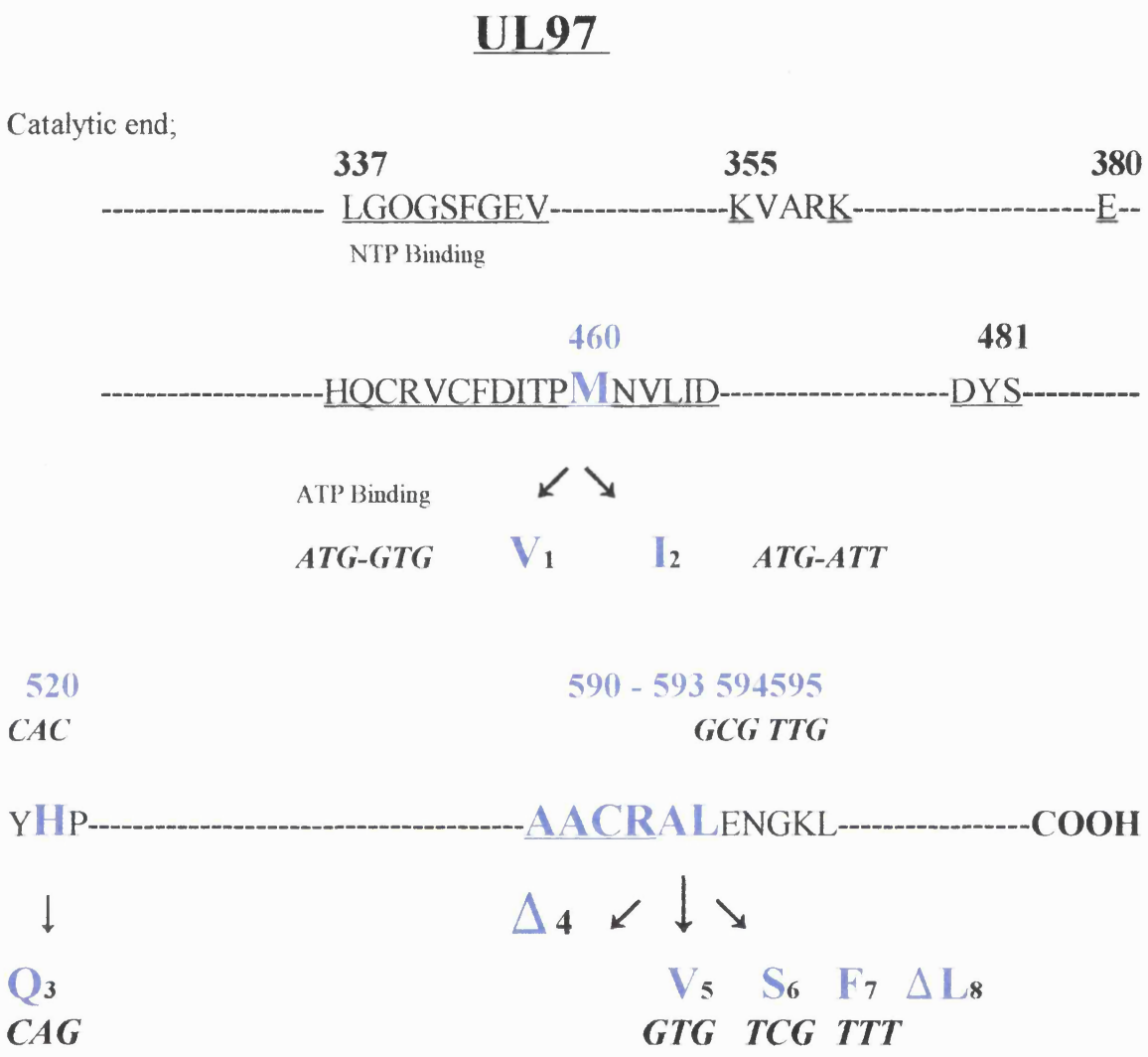
The early studies of HCMV GCV resistant mutants indicated that the selective action of GCV depended on the monophosphorylation of GCV in infected cells (Stanat *et al.* 1991). To identify the enzyme responsible for GCV phosphorylation Sullivan *et al* studied the viral genome of the laboratory derived HCMV mutant 759rD100 known to be deficient in GCV anabolism (Sullivan *et al.* 1992). In order to map the site of the resistance within the viral genome the mutant was cloned into a set of nine cosmids and subsequent marker transfer studies identified two cosmids (pC7537 and pC7595) that transferred GCV resistance to wild type Ad169. In the cosmid pC7537 the resistance was mapped to the viral DNA *pol* (UL54) with GCV phosphorylation unaffected. However, the GCV resistant recombinant isolated from pC7595 had intact DNA polymerase function but an IC_{50} ten times higher than AD169. Subsequent work was therefore directed to the 13 kb overlap unique to pC7595. In this

overlap a 2.6 kb region containing the complete UL97 ORF was highlighted. On DNA sequencing the UL97 ORF a 12bp deletion was found resulting in a four amino acid deletion of residues Ala-Ala-Cys-Arg (amino acids 590-3). The viral specificity of UL97 was proven by Littler *et al* by producing antisera from purified UL97 which then reacted on western blotting with UL97 and a protein from HCMV infected cells (Littler *et al.* 1992). In order to ascertain the ability of UL97 to phosphorylate GCV, Littler *et al* assayed extracts from *Escherishia coli* BL21 cells containing a plasmid with either the wild-type or mutant truncated UL97 insert. Extracts from BL21 cells expressing UL97 efficiently phosphorylated GCV whereas control extracts did not. UL97 homologues are encoded by HSV, VZV, EBV, HHV6 and 7 suggesting a conservation of function among herpesviruses (Sullivan *et al.* 1992). HCMV UL97 has been successfully expressed by a recombinant vaccinia virus (Metzger *et al.* 1994). This insertion of UL97 into vaccinia made the recombinant vaccinia susceptible to GCV. Temporal expression of the UL97 gene product has shown that the protein, which appears to show nuclear localisation, can first be identified 16 hours pi (Michel *et al.* 1996). This expression was blocked by inhibitors of viral DNA replication suggesting that UL97 is a late protein. Interestingly, Michel *et al* found that GCV itself down-regulated the expression of UL97. The natural function of the UL97 protein has yet to be established but it appears that UL97 does not phosphorylate natural nucleosides (Michel *et al.* 1996). Further clarification of the natural function of the UL97 protein in the resting viral life cycle would greatly enhance the direction of new anti-viral therapies.

The 12 bp deletion resulting in the loss of the 4 amino acids (590-593) has only been found in one clinically resistant HCMV strain isolated from a patient (Chou *et al.* 1995b). Another clinical isolate was found to have a three bp deletion resulting in the loss of leucine at 595 and

marker transfer studies confirmed the genotypic resistant phenotype of this deletion mutant (Baldanti *et al.* 1995). Several groups have now extensively analysed the UL97 coding sequence both from clinical isolates and directly from plasma samples from patients on long term anti-viral therapy (Chou *et al.* 1995a; Wolf *et al.* 1995). To date 6 coding mutations in the UL97 sequence have been shown to confer GCV resistance to Ad169 using marker transfer experiments (Chou *et al.* 1995a; Wolf *et al.* 1995; Lurain *et al.* 1994; Hanson *et al.* 1996; Baldanti *et al.* 1995) (see Figure 1.4).

Mutations involving the amino acids 460, 594 and 595 are thought to account for over 85% of all genotypic phenotypes of HCMV UL97 resistance to GCV (Chou *et al.* 1995b). The highly conserved methionine at codon 460 lies in UL97 subdomain VI and is thought to be implicated in nucleotide binding, whereas the amino acids AACRL, at codons 590 to 595, are in subdomain IX and involved in substrate recognition. The relationship between genotypic mutations and phenotypic resistance using marker transfer studies is shown in Table 1.4.



M ; mutation confirmed by marker transfer studies
K; catalytic lysines
E; conserved E in protein kinases
D; conserved within protein kinases & phosphotransferases

1. Chou 1995; **2.** Lurain 1994; **3.** Hanson 1995; **4.** Sullivan 1992; **5.** Chou 1995
6 & 7. Chou 1995 & Wolf 1995; **8.** Baldanti 1995

Figure 1.4: The UL97 open reading frame illustrating mutations that have been shown to confer resistance to Ad169 using marker transfer studies.

Table 1.4: IC_{50} and % anabolism of ganciclovir for Ad169 and recombinant viruses carrying different UL97 mutations.

Resistance mutation	GCV IC_{50} µM Recombinant	GCV IC_{50} µM Ad169 control	% Anabolism*	Reference
M460V	5.2	0.74	31	Chou 1995
H520Q	12.7	1.20	21	Hanson 1995
A594V	5.9	0.55	20	Chou 1995
L595S	6.4	0.55	19	Chou 1995
L595F	33	2.5	N/A	Wolf 1995
	GCV EC_{50} µM Recombinant	**GCV EC_{50} µM Ad169 control**	**%Anabolism**	
M460I	200	10	10	Lurain 1994

* compared to GCV phosphorylation activity as % of Ad169

HCMV resistance to ganciclovir can also arise from mutations in the DNA polymerase gene (Stanat *et al.* 1991). Analysis of the laboratory mutant 759rD100 found an alanine to glycine substitution at amino acid position 987 within the conserved V region that also conferred resistance to other HCMV DNA pol inhibitors such as HPMPC and HPMPA (Sullivan *et al.* 1993). Other substitutions in the conserved region IV have also been shown to confer resistance to other DNA pol inhibitors (Lurain *et al.* 1992). Recently the molecular basis of the double resistance to both GCV and foscarnet has been described from the clinical isolates of AIDS patients on long term sequential courses of both drugs (Baldanti *et al.* 1996). Two separate amino acid changes were found, one in UL97 causing GCV resistance and one in domain II of DNA pol causing foscarnet resistance. The recombinant strain carrying just the DNA pol mutation was sensitive to GCV suggesting that the resistance was due to two separate mechanisms. The recombinants expressing the UL54 mutations in amino acids 715 and 700 exhibited a slow growth phenotype and a reduction in yield of infectious virus suggesting that this region of DNA pol may play an important role in HCMV replication.

The resistance patterns of HCMV to foscarnet have been less well characterised than for ganciclovir. HCMV foscarnet resistant strains that also show cross resistance to other DNA polymerase inhibitors have been demonstrated in both the laboratory and clinical settings (Sullivan *et al.* 1993; Tatarowiczk *et al.* 1992; Lurain *et al.* 1992). The molecular basis for HCMV foscarnet resistance appears to be predominately via mutations at Val-715-Met or Thr-700-Ala in domain II of HCMV DNA pol (Baldanti *et al.* 1996). Both these mutations were shown by marker transfer studies to confer foscarnet resistance to Ad169 which remained sensitive to ganciclovir and cidofovir. Resistance to cidofovir has not yet been extensively studied at the molecular level but preliminary data indicate that polymerase mutations at new

amino acid sites such as 301, 501, 503 and 987 may be important (Cherrington *et al.* 1996). Therefore, it appears that DNA polymerase mutations conferring resistance to GCV, PFA and CDV may all occur at different amino acid residues and cross resistance is due to multiple mutations in UL54. The only report of a single DNA polymerase mutation that can confer cross-resistance to all three agents is the glycine-to-alanine substitution at amino acid position 987 within the conserved region V of the DNA pol of the laboratory derived HCMV mutant 759rD100 (Sullivan *et al.* 1993). This mutation has not been found in clinical isolates.

1.28 Aims of the Thesis

The aim of this thesis was to study the natural history of HCMV infection in the HIV infected individual comparing the relationship between becoming HCMV PCR positive, subsequent changes in HCMV load and genotypic changes in UL97 with each patients disease course. The thesis had three major aims:

The first aim was to prospectively recruit a cohort of AIDS patients with HCMV retinitis, and relate HCMV viral load with response to therapy and disease progression. Longitudinal follow-up then allowed investigation of the significance of HCMV monitoring (by PCR) during induction and maintenance therapy and the optimisation of each patients clinical management.

The second aim was to correlate the detection of UL97 mutations with the clinical course of the patient. This involved the development of a PCR for UL97 and a rapid technique (point mutation assay) for screening PCR products for UL97 mutations. As the UL97 mutant strains could not be cultured from peripheral blood, another method for studying the phenotypic function of these mutations was needed and expression of UL97 in a baculovirus expression system was chosen.

The third aim was to identify patients with HCMV viraemia (by PCR) and determine if they were more at risk of retinitis and to offer them regular ophthalmological screening. The validation of a systemic marker for patients at a high risk for HCMV disease would therefore enable the future study of pre-emptive therapeutic agents in a controlled setting.

CHAPTER 2

THE INFLUENCE OF CYTOMEGALOVIRAL LOAD ON RESPONSE TO GANCICLOVIR INDUCTION THERAPY, TIME TO RECURRENCE AND SURVIVAL IN AIDS PATIENTS WITH HCMV RETINITIS

2.1 Introduction

In congenitally infected infants and allograft recipients the importance of viral load in human cytomegalovirus pathogenesis has been detailed in studies using conventional cell culture methods (Stagno *et al.* 1975) and, more recently, molecular methods (Saltzman *et al.* 1992; Landry and Ferguson, 1993). Studies have shown that renal transplant recipients with symptomatic HCMV infection have significantly higher levels of viruria than those patients who remained asymptomatic (Fox *et al.* 1995). In AIDS patients, Rasmussen *et al* have applied a colorimetric quantitation assay to study patients with HCMV retinitis and those with HCMV infection but no evidence of end organ HCMV disease (Rasmussen *et al.* 1995). They found that copy numbers of HCMV DNA in blood were significantly higher in those patients with retinitis but there was no comment on the influence a patients viral load had on their subsequent clinical course (Rasmussen *et al.* 1995). There have been no studies that have prospectively addressed the issue of the natural history of HCMV load in HIV infected patients. I have applied a quantitative-competitive PCR method to measure HCMV load in whole blood and urine in a group of AIDS patients recruited prospectively as they presented with HCMV retinitis. These data have allowed me to address the inter-relationships between HCMV load, response to therapy, time to progression, and survival.

The basis of the methodologies described in this chapter is the polymerase chain reaction (PCR). This technique has allowed a major advance in the field of molecular biology enabling molecular techniques to be carried out in a real time setting to enhance clinical medicine. PCR is an *in vitro* method for primer directed enzymatic amplification of a specific DNA target. Two oligonucleotides which flank the DNA target sequence are used to prime the activity of a DNA polymerase enzyme. This pair of oligonucleotide "primers"

are specific to the DNA sequence of interest and are submitted to repeated cycles of high temperature denaturation, primer annealing and extension of the annealed primers by a DNA polymerase enzyme. During each of these cycles the amount of DNA present in the previous cycle is potentially doubled leading to an exponential increase (2^n, n being the number of cycles) of the DNA target sequence originally present in the reaction. The first description of DNA amplification used the *Klenow* fragment of DNA polymerase I for primer extension. However, the high temperatures needed to denature the DNA also inactivated the enzyme. The use of thermostable DNA polymerases, such as that of the bacterium *Thermus Aquaticus* (*Taq*), which remains stable and enzymatically active at high temperatures, has been crucial in the development of the PCR technique (Saiki *et al.* 1988). The commercially available *Taq* polymerase is a recombinant form of the natural enzyme, which lacks its endonuclease and 3' to 5' exonuclease activities but retains its 5' to 3' polymerisation-dependent replacement activity. *Taq* polymerase is active above temperatures of 72°C, a temperature which has the benefit of allowing high specificity of the PCR by dissociating non-specific annealing of primers, therefore allowing only primers bound to their specific complementary sequence to remain intact for the extension reaction.

One of the benefits of the use of thermostable DNA polymerase was to limit the manipulation of the reaction mixture during the procedure and therefore reduce the possibilities of contamination and increase automation. Indeed as PCR is an extremely sensitive technique contamination with target DNA, not originally present in the sample analysed, has been the main problem in the use of PCR as a research and diagnostic tool. Contamination can be limited with careful adherence to simple rules such as fastidious application of sterile technique and the use of a different room for each of the three steps in

setting up a PCR (Kwok and Higuchi, 1989). Therefore, the reaction components are assembled in a clean room where the presence of any target DNA is strictly prohibited and operators are fully clothed in sterile paper gowns and gloves. Target DNA is added in a separate area and the PCR assays are then run on automated thermo-cycling machines (Hybaid) in a third distinct room. All of the PCR's described in this thesis were conducted using this three room technique.

The success of a PCR depends on the careful balance between the maximum amplification of the specific target required whilst preventing the amplification of non specific products that may contribute to a false positive result. Therefore, each PCR needs to be optimised according to the specific target that is to be amplified. Reaction conditions that can be altered to optimise the PCR include the annealing temperature, the concentration of free magnesium ions and the concentration of the primers. All of these steps were taken whilst optimising the PCRs described in detail in this thesis.

This chapter describes the application of a quantitative-competitive PCR to the study of HCMV viral load in AIDS patients with HCMV retinitis.

2.2 Methods

2.2.1 Study Population

All HIV-seropositive patients who attended the AIDS clinic at the Royal Free Hospital from 1 September 1993 with a diagnosis of HCMV retinitis were asked to participate in the study. Patients who had received ganciclovir or foscarnet therapy previously for other manifestations of HCMV disease were excluded. Samples of blood (10 ml in either preservative-free heparin or citrate filled tubes) and urine were collected before treatment (on the day of diagnosis of retinitis), at the end of the induction period (day 21), and then monthly thereafter. Additional samples of blood and urine were collected at times of retinitis progression.

2.2.2 Criteria for Diagnosis of HCMV Retinitis

Cytomegalovirus retinitis was diagnosed by a fully qualified ophthalmologist using indirect ophthalmoscopy through dilated pupils and applying criteria set out by Heinemann (Heinemann, 1992). Patients were reviewed every two weeks for one month after the initial diagnosis of retinitis and then on a monthly basis unless a patient presented with new or worsening visual symptoms. Progression was determined clinically by the ophthalmologist, who was aware of the patients current anti-HCMV treatment but was unaware of the results of all virological parameters. Where possible fundus photographs were taken at every visit.

2.2.3 Treatment

All patients received ganciclovir for induction therapy and as primary maintenance therapy. Induction therapy was given as a 5 mg per kilogram intravenous (iv) infusion twice daily for 21 days. At the beginning of the study, all patients received ganciclovir iv 5 mg per

kilogram once daily, 5 days a week as maintenance therapy. However, from December 1993, patients were given the option to receive oral ganciclovir as maintenance therapy either as part of a trial (Syntex/Hoffman la Roche, UK Ltd; randomized to 3,000 mg or 6,000 mg total per day) or, more recently, as open label oral ganciclovir 3,000 mg per day. Any patient whose retinitis progressed on maintenance ganciclovir was re-induced with high dose iv ganciclovir for 2 weeks then restarted on his/her previous maintenance regimen. If a patient's retinitis continued to progress or if the patient was intolerant of further ganciclovir therapy, he/she was switched to foscarnet (90mg/kg bd iv induction; 90mg/kg od iv maintenance), and then subsequently to cidofovir (5mg/kg weekly for two weeks induction; 5mg/kg fortnightly thereafter) if retinitis progressed or the patient became intolerant of foscarnet.

2.3 DNA Extraction From Clinical Samples

DNA was extracted from whole blood samples using ion-exchange chromatography in a commercial QIAamp blood PCR kit supplied by Qiagen, UK. A 200µl aliquot of whole blood was treated with 200µl lysis buffer and 25µl Qiagen proteinase (19.23 mg/ml). The mixture was vortexed and incubated at 65^{0}C for 30 min prior to being adsorbed onto an ion exchange column in a 1.5ml microcentrifuge tube containing a silica matrix bed and centrifuged (Jouan MK14.11, Jouan, UK) at 6000g for 1 min. The DNA that had adsorbed to the column was subsequently washed twice with Qiagen buffers containing guanidinium hydrochloride (formula not available) and then eluted in 200µl sterile distilled water (SDW) (pre-heated to 70^{0}C) by centrifugation at 6000g for 1 min. If the sample was to be used immediately for a PCR it was heated to 90^{0}C to inactivate the proteinase, otherwise it was stored at -70^{0}C until use.

Samples of vitreous fluid were centrifuged at 1600g for 15 min, the supernatant was removed and boiled for 10 min before being stored at -70^{0}C until use.

Urine and CSF were analysed neat in all PCR reactions and were not subjected to further extraction.

All target specimens were processed at neat and 1 in 20 dilutions.

2.4 Amplification of gB by Qualitative PCR

2.4.1 Oligonucleotide Primers Used for gB PCR

The sequence of primers used to amplify a 149 bp product from the gB region of HCMV in both qualitative and quantitative PCR reactions were as follows:

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

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

Both sequences were taken from the laboratory prototype strain Ad169 and correspond to nucleotides 81683-81707 (gB1) and 81559-81820 (gB2) (Chee MS. and Bankier AT. 1990). The oligonucleotides were synthesised and HPLC purified commercially and supplied by Cruachem (UK).

2.4.2 Optimisation of gB PCR

This PCR had been fully optimised for concentration of magnesium ions, primer concentrations and cycling conditions by Fox 1992 (Fox *et al.* 1992). The optimum working concentration for magnesium chloride in the reaction was 2mM $MgCl_2$ with 100ng of each of the gB primers. These optimised components were then added to a reaction mixture of

10x PCR buffer (10x NH_4 buffer) containing 250mM Tris-HCl pH 8.4, 170mM ammonium sulphate, 100mM β-mercaptoethanol, 0.02% (w/v) gelatin, and 200µM of each dNTP. Finally, 2.5 units of *Taq* polymerase were added to each reaction mixture.

The deoxynucleotide triphosphates were made up as follows (all supplied from Promega, UK):

dNTP mixture:	6.25 mM	dATP (deoxyadenosine triphosphate)
	6.25 mM	dCTP (deoxycytosine triphosphate)
	6.25 mM	dGTP (deoxyguanosine triphosphate)
	6.25 mM	dTTP (deoxythymidine triphosphate)

Therefore the PCR reaction mixture for one tube was made up as follows:

10µl 10x NH4 buffer
4µl $MgCl_2$ (50mM)
3µl dNTP mixture (200µM each)
1µl gB1 (100ng)
1µl gB2 (100ng)
0.5µl *Taq* polymerase (2.5 units)
75µl SDW

=95µl total volume

The volume of SDW in the reaction was adjusted according to the amount of target DNA to be added. The reaction mixture is finally overlayed with 100µl of mineral oil (molecular biology grade, Sigma) to minimise contamination and prevent aerosol spread. All of the above procedures were carried out in the "clean PCR room" using aerosol resistant tips, sterile gowns and gloves.

Target DNA (5µl) was added in a separate room in a class II microbiological safety cabinet (British Standard 5726, 1992), making the total reaction mixture up to 100µl in a 0.5ml Eppendorf tube. 5µl SDW only was added to every fifth tube to act as a negative control and

one tube had DNA extracted from Ad169 as the positive control. Only one PCR tube was open at any one time and latex gloves were changed frequently to minimise contamination. The PCR reaction was performed in a Hybaid TR2 thermal cycler (Hybaid, UK) in a third designated room. The cycling conditions that maximised target amplification whilst minimising non-specific product production were as follows:

Stage 1: 1 cycle:	94^{0}C 4 min
	60^{0}C 2 min
	72^{0}C 2 min
Stage 2 : 35 cycles:	94^{0}C 1 min
	60^{0}C 1 min
	72^{0}C 1 min
Stage 3 : 1 cycle:	94^{0}C 1 min
	60^{0}C 1 min
	72^{0}C 10 min

2.4.3 Visualisation of PCR Amplicons on Agarose Gel

The PCR products (amplicons) from each experiment were confirmed by agarose gel electrophoresis. Three grams of agarose were weighed into a Duran bottle and melted in 100ml of 1xTBE for three minutes in a 750W microwave oven at full power. Once the dissolved agarose had cooled to below 50^{0}C, 0.5µl of a 10 mg/ml ethidium bromide solution (Sigma) was added and the agarose was poured into a gel frame with a 15-20 well comb (depending on the number of PCR reactions to be analysed) and left to set. After the comb had been removed and the gel placed in an electrophoresis tank, 10µl of each PCR reaction (mixed with 5µl loading buffer of 30% glycerol, 0.1% bromophenol blue in distilled water) was added to each well. A PCR marker (1000bp to 100bp, Promega) was also run to confirm the mobility of the amplicons. The gel was electrophoresed in 1x TBE at 150V for

approximately 30 minutes and then visualised on a transilluminator and photographed using Polaroid film.

Buffer used in agarose gel electrophoresis:

10x TBE: 432g Tris base (Sigma)

220g boric acid (Sigma)

37.2g EDTA (Sigma)

made up to 4 litres with distilled and deionized water.

2.5 Quantitative-competitive PCR for HCMV

All samples that were qualitatively positive for HCMV were subsequently quantified for the amount of HCMV DNA present. The same whole blood DNA and urine extracts were used for quantification. The method used was described by Fox in 1992 and involves the addition of a control sequence that closely resembles the actual target sequence (Fox *et al.* 1992). This co-amplification method of adding control sequence to each PCR mixture can compensate for local variations in PCR tubes and the presence of inhibitory substances. Therefore, for each PCR a pre-determined amount of control sequence was added along with the target DNA. In order to differentiate the target and control amplimers a unique *Hpa*I restriction site was added by PCR mutagenesis to the control sequence at bases 77 and 78 by changing the original GG to TT (Fox *et al.* 1992). The subsequent 149 bp control product was purified and cloned into pUC13. After co-amplification of target and control sequences in the PCR, the products are subjected to enzymatic digestion using *Hpa*I and the digested products can be separated using polyacrylamide gel electrophoresis (PAGE). The control sequence is digested into 77 and 72 bp fragments whilst the target amplicons migrate more slowly at 149 bp. The use of [γ^{32}P]ATP to label the gB2 5' OH allows the detection of these

bands by autoradiography and scanning densitometry.

2.5.1 Optimisation of Quantitative gB PCR

The quantitative-competitive PCR (QCPCR) had been fully optimised for concentration of magnesium ions, primer concentrations and cycling conditions by Fox *et al*, 1992 (Fox *et al.* 1992). The optimum working concentration for magnesium chloride in the reaction was 2mM $MgCl_2$ and for the gB primers was 100ng for each primer. These optimised components were then added to a reaction mixture of 10x PCR buffer as described in section 2.4.2.

The gB2 primer was 5' phosphorylated as follows; 500ng gB2 was mixed with 20µl 5x polynucleotide kinase buffer (350mM Tris-HCL, pH 7.4, 50mM $MgCl_2$, 500mM KCl and 5mM β-mercaptoethanol), 2 units polynucleotide kinase (10u/µl Gibco BRL, UK) and 10µl [γ^{32}P]ATP (100 µCi, Amersham UK) and made up to 100µl with 66µl SDW. The mixture was placed in a 37^{0}C water bath for 30 min and the reaction was then stopped by placing the reaction mixture directly at -70^{0}C until use. All reactions involving radio labelled [γ^{32}P]ATP were carried out following strict departmental radiation safety measures including the disposal of all radioactive waste in designated areas.

The reaction mixture for one PCR was as follows;

10µl	10x NH4 buffer
4µl	$MgCl_2$ (50mM)
3µl	dNTP mixture (200µM each)
1µl	gB1 (100ng)
1µl	gB2 (100ng)
0.5µl	gB2 [γ^{32}P]ATP labelled (5ng/µl)
0.5µl	*Taq* polymerase (2.5 units)
73µl	SDW

=93 µl	total volume

The reaction mixture was overlayed with 100 µl of mineral oil. In the separate PCR set up laboratory 5µl of target DNA was added to each PCR reaction and 5µl of SDW was added to the negative controls. After target had been added to all the reactions a known amount of control sequence was added to each reaction. In the first instance 1000 copies were usually added to each reaction. In general this concentration allowed the quantification of the majority of samples. Depending on the results obtained, different amounts of control could be added subsequently. All samples were analysed in triplicate and at different concentrations to yield an accurate quantitation, aiming for an optimal ratio of target to control between 60 to 40%. The PCR cycling conditions were identical to those detailed in section 2.4.2 and the amplicons were visualised on a 3% agarose gel as detailed in section 2.4.3.

2.5.2 Digestion of Target/control gB Amplicons

Following electrophoresis of the QCPCR to confirm successful amplification, the amplicons were digested with *Hpa*I. 10µl of each PCR reaction product was digested with 0.6µl of *Hpa*I (5 U/µl), 2µl of 10 x buffer (200mM Tris-HCl pH 7.4, 50mM $MgCl_2$, 500mM KCl) and 7.4µl of SDW to a total volume of 20µl. The reaction was incubated at 37^0C for a minimum of 90 min. The digested products were then mixed with loading buffer and electrophoresed on a 12% polyacrylamide gel (6ml 40% polyacrylamide, 2ml 10xTBE, 12ml SDW, 140µl 10% w/v ammonium persulphate (APS) and 14µl N,N,N',N' tetramethylethylenediamine (TEMED)) at 45V until the bromophenol blue dye had run off the gel. The gels were then placed in 1xTBE, containing 5µl of a 10 mg/ml ethidium bromide solution to enable visualisation of the DNA, and photographed using a transilluminator. The digested products were then detected by autoradiography using

Hyperfilm-MP (Amersham, UK) placed directly onto the gels in an X-ray cassette. Scanning densitometry using a Shimazdu CS 9001 PC dual wave scanning densitometer was used to compare the different signals from target DNA and the known amount of control DNA.

2.6 Statistics

Patient follow-up was censored on 31 December 1995. The relationships between PCR status (positive/negative) and HCMV load in blood and urine (above/below the median initial viral load of all patients included in the study) and the time to first progression of retinitis or death were compared graphically using Kaplan-Meier plots and log rank tests. The decision to divide patients according to the median viral load of the cohort was used *a priori* to circumvent *post-hoc* analytical criticisms. Time to first progression of retinitis was considered from the date of starting maintenance therapy, whereas survival was considered from the date of diagnosis of the HCMV retinitis. Multivariate analyses using a proportional hazards model (Cox and Oakes, 1984) were carried out to quantify the effect of HCMV load on progression rates of retinitis and to assess whether this effect could be accounted for by differences in maintenance therapy administration (oral/iv), CD4 count, or age of patient. For these analyses, viral load, CD4 count and age were treated as continuous variables. The analysis was also repeated using categorical variables (above/below median values), although the results were essentially unchanged.

2.7 Results

2.7.1 Patients

Between 1 September 1993 and 31 December 1995, 52 patients developed HCMV retinitis of whom 47 were eligible for recruitment into this study (2 patients had received ganciclovir previously and three patients were lost to follow-up after completing induction therapy). Of the 47 patients, one declined to give regular blood samples and one refused treatment but agreed to have regular samples taken for HCMV viral load measurements and attend for ophthalmological follow-up including indirect fundus photography. In total, 45 patients were available for analysis: 37 men, (of whom 34 (92%) were homosexual/bisexual, 2 intravenous drug users, one heterosexual) and 8 women. The median age of the cohort was 34 years (range 26 - 62 years) and the median CD4 count at diagnosis of retinitis was 10 cells/μl (range 0 - 150 cells/μl). The Kaplan-Meier estimate of the median time to first progression of retinitis in the cohort was 78 days and median time to death was 8.7 months (265 days).

2.7.2 Treatment

After diagnosis, 43 patients were treated with a 21-day induction course of iv ganciclovir at a dose of 5 mg per kilogram twice daily. Two patients had peripheral unilateral retinitis diagnosed at elective screening that was deemed inactive at day 14 and a decision was made to go straight onto maintenance therapy. Stabilisation of HCMV retinitis was observed in all 45 patients following induction therapy.

Thirteen patients received iv ganciclovir as maintenance therapy and thirty-two patients received oral ganciclovir maintenance therapy.

2.8 Qualitative PCR Results

Thirty-nine (87%) of the 45 patients were PCR positive at diagnosis either in blood or urine. Forty-six per cent of these patients were PCR positive in blood only, 23% in urine only, and 31% in both. Six patients were PCR negative for HCMV in both blood and urine at diagnosis of retinitis. All 6 of these PCR negative patients were IgG seropositive for HCMV. Five of these 6 patients remained PCR negative throughout the study and their retinal lesions responded well to ganciclovir induction therapy although clinical progression of the retinitis, during maintenance treatment, was observed unilaterally in 4 of these patients. One patient became HCMV PCR positive after 17 months on maintenance therapy that coincided with bilateral spread of HCMV retinitis to the previously uninfected fellow eye.

2.9 Quantitative PCR Results

Table 2.1 shows the baseline age, CD4 count, p24 antigen level where available and HCMV load for all 45 patients. In the 39 PCR positive patients, the median pre-therapy HCMV load in blood was 4.95 $\log_{10}$ genomes/ml (range 3.6 - 7.05 $\log_{10}$ genomes/ml) and in urine was 4.90 $\log_{10}$ genomes/ml (range 4.45 - 6.46 $\log_{10}$ genomes/ml). Figure 2.1 shows the titration of clinical samples with different copy numbers of control sequence using polyacrylamide gel electrophoresis to separate the radiolabelled target/control amplicons after digestion with *Hpa I*.

Table 2.1: Baseline demographics of all 45 patients in the cohort with their HCMV loads in blood and urine and at presentation.

Pt no.	Initial	HCMV blood	HCMV urine	D21 B	D21 U
1	PM	4.15	ND	-	
2	PB	3.60	ND	-	
3	DM	5.64	ND	-	
4	MT	5.27	ND	**4.64**	
5	DJ	5.89	ND	**5.18**	
6	DW	6.18	ND	**4.30**	
7	PR	5.39	ND	-	
8	DL	5.28	ND	-	
9	TE	4.08	-	-	
10	MG	3.73	-	-	
11	TH	3.99	-	-	
12	TP	-	-	-	
13	JS	ND	4.54	-	
14	AH	-	4.71	-	**3.84**
15	MS	-	-	-	
16	JFL	4.95	ND	-	
17	MCP	-	-	-	
18	TD	4.48	-	-	
19	JB	-	-	-	
20	ET	4.34	-	-	
21	MP	5.25	5.36	-	-
22	PS	5.87	-	-	
23	AB	-	4.96	-	-
24	MC	-	5.83	-	-
25	CK	4.65	4.58	-	-
26	JH	-	5.28	-	-
27	MA	-	4.79	-	-
28	RJ	4.72	-	-	
29	RM	5.76	-	-	
30	SH	4.29	-	-	
31	AG	4.55	4.83	-	-
32	GN	-	-	-	
33	DS	-	4.54	-	-
34	IR	6.61	6.15	**4.04**	**3.91**
35	IB	5.18	5.65	-	-
36	FP	4.55	5.75	-	-
37	LR	4.75	4.62	-	-
38	BP	6.41	6.34	-	**4.16**
39	LM	7.05	6.46	-	-
40	MY	-	4.72	-	-
41	LW	-	4.45	-	-
42	SB	-	-	-	-
43	JO	5.77	5.62	-	-
44	TLC	4.82	4.90	-	-
45	NTP	4.85	4.58	-	-

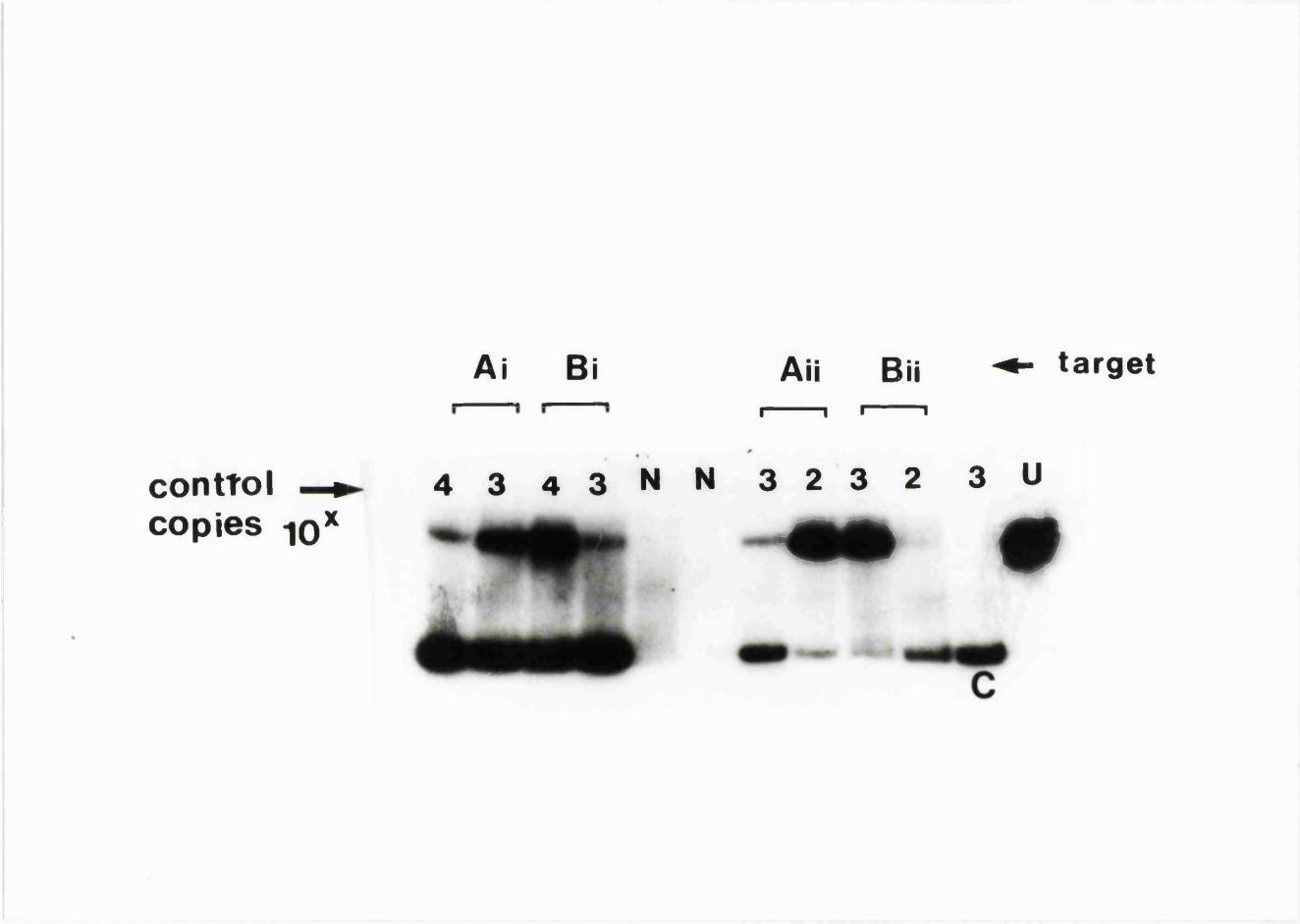


Figure 2.1: Autoradiograph of the *Hpa*I digested PCR products showing the titration of two clinical samples (A and B) with different control copy numbers from 10,000 to 100 copies. N= negative control; U= uncut positive control; C= control post digestion.

2.9.1 HCMV Load and HCMV Retinitis in the Absence of Treatment

One patient who was found to have a peripheral lesion of HCMV retinitis in his left eye after complaining of flashing lights, declined any treatment for his retinitis but agreed to take part in this study. At presentation with HCMV retinitis the HCMV load in blood and urine was 5.28 $\log_{10}$ genomes/ml and 7.22 $\log_{10}$ genomes/ml respectively. The changes in HCMV load (and the development of other HCMV related diseases) over a 7 month period are shown in Figure 2.2 and the concomitant progression of the HCMV retinitis is shown in Figure 2.3.

The HCMV load in blood at diagnosis of retinitis was comparable to the median viral load of the cohort. However, the viral load in urine was higher than the cohorts median viral load in urine (7.22 $\log_{10}$ genomes/ml vs 4.90 $\log_{0}$ genomes/ml). A urine sample collected 6 months prior to this diagnosis had an HCMV load of 4.87 $\log_{10}$ genomes/ml showing that HCMV load in urine had increased prior to the development of retinitis. The overall increase in HCMV viral load in the blood and urine from initial diagnosis to death (7 months) was 3600-fold. In the 7 months during this increase in HCMV DNAemia, the patient suffered a gamut of HCMV related diseases resulting in blindness, adrenal insufficiency and, ultimately, death from HCMV encephalitis. The serial retinal photographs shown in Figure 2.3 illustrate the rapidity of the retinal progression of untreated disease from a small, peripheral, unilateral lesion to bilateral end stage disease leaving no residual uninfected viable retina (Figures 2.3 a&b and g&h). Indeed, the rate of expansion of the peripheral lesions resulted in a greater than three fold increase in size over one month (Figures 2.3 b&d). The rapid "brush-fire" progression of the initial lesion and its coalescence with the central lesion within three months are aptly illustrated in Figures 2.3 b&f.

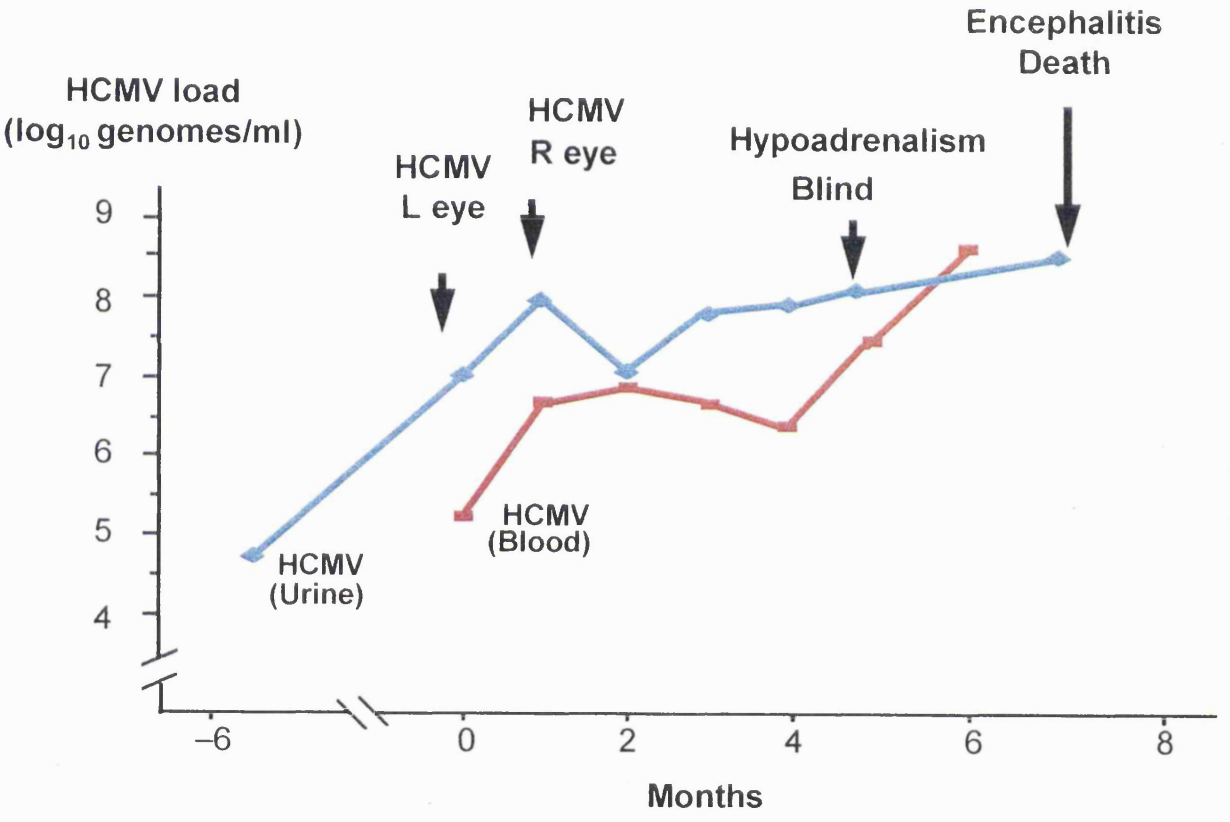


Figure 2.2: Changes in HCMV load in blood and urine following a diagnosis of HCMV retinitis in absence of anti-HCMV therapy. The clinical course of the patient is also illustrated.

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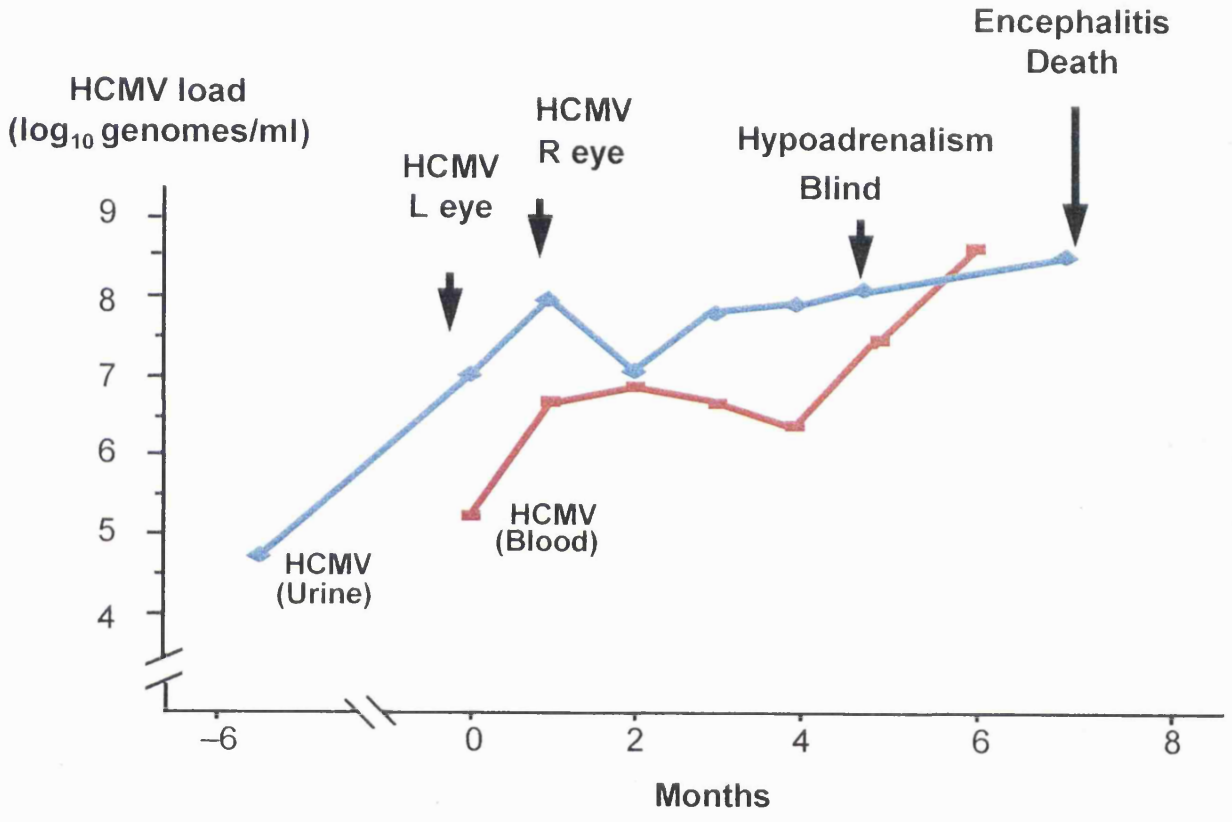

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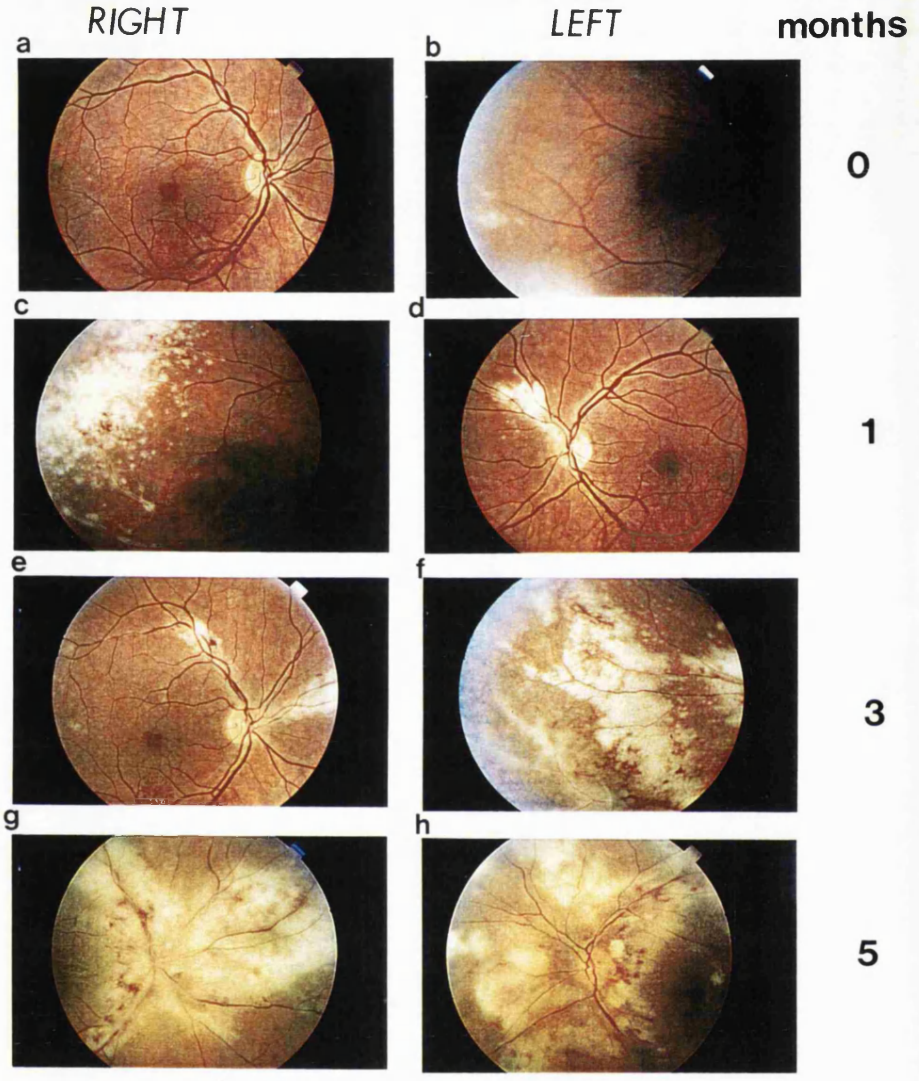

Figure 2.3: Serial retinal photographs illustrating the rapid progression of HCMV retinitis in the absence of treatment from a small, peripheral unilateral lesion (2.3b), to the fellow eye (2.3c) and then to both optic discs (2.3e&f). Eventually, following "brush-fire" progressions (2.3f), there is very little uninfected viable retina to be seen (2.3g&h).

2.9.2 HCMV Load and Response to Ganciclovir Induction Therapy

Following induction therapy with ganciclovir, 33 of the 39 PCR positive patients (85%) subsequently became PCR negative at day 21 (see Figure 2.4). As the detection limit for HCMV in blood or urine in this QCPCR assay is approximately 200 genomes/ml (2.3 $\log_{10}$ genomes/ml), this corresponds to a mean reduction in viral load of 2.8 log genomes/ml (range 1.3 - 4.7) in blood and 2.9 $\log_{10}$ genomes/ml (range 2.1 - 4.2) in urine. Six patients still had detectable HCMV DNA either in blood (n=3), urine (n=2) or both fluids (n=1) after completing induction therapy. After one month of maintenance therapy, 5 of these patients had become HCMV PCR negative; the remaining patient having died of *Pneumocystis carinii* pneumonia soon after completing induction therapy.

Pre-treatment viral loads in blood of the 6 patients who remained HCMV positive after induction therapy were significantly higher than the 33 patients who became PCR negative after induction therapy (median load was 6.04 $\log_{10}$ genomes/ml compared to 4.82 $\log_{10}$ genomes/ml for those who responded to induction therapy, p=0.018, Figure 2.5). The median viral load in urine of the patients who did not responded to induction therapy was greater than for those who did respond but this was not significant (6.15 $\log_{10}$ genomes/ml compared to 4.87 $\log_{10}$ genomes/ml, p>0.05). The median time to progression in the patients who remained PCR positive was 40 days compared to 78 days for the remaining 33 patients who were PCR negative after induction therapy. However, due to the small number of patients in each group, this difference was not statistically significant.

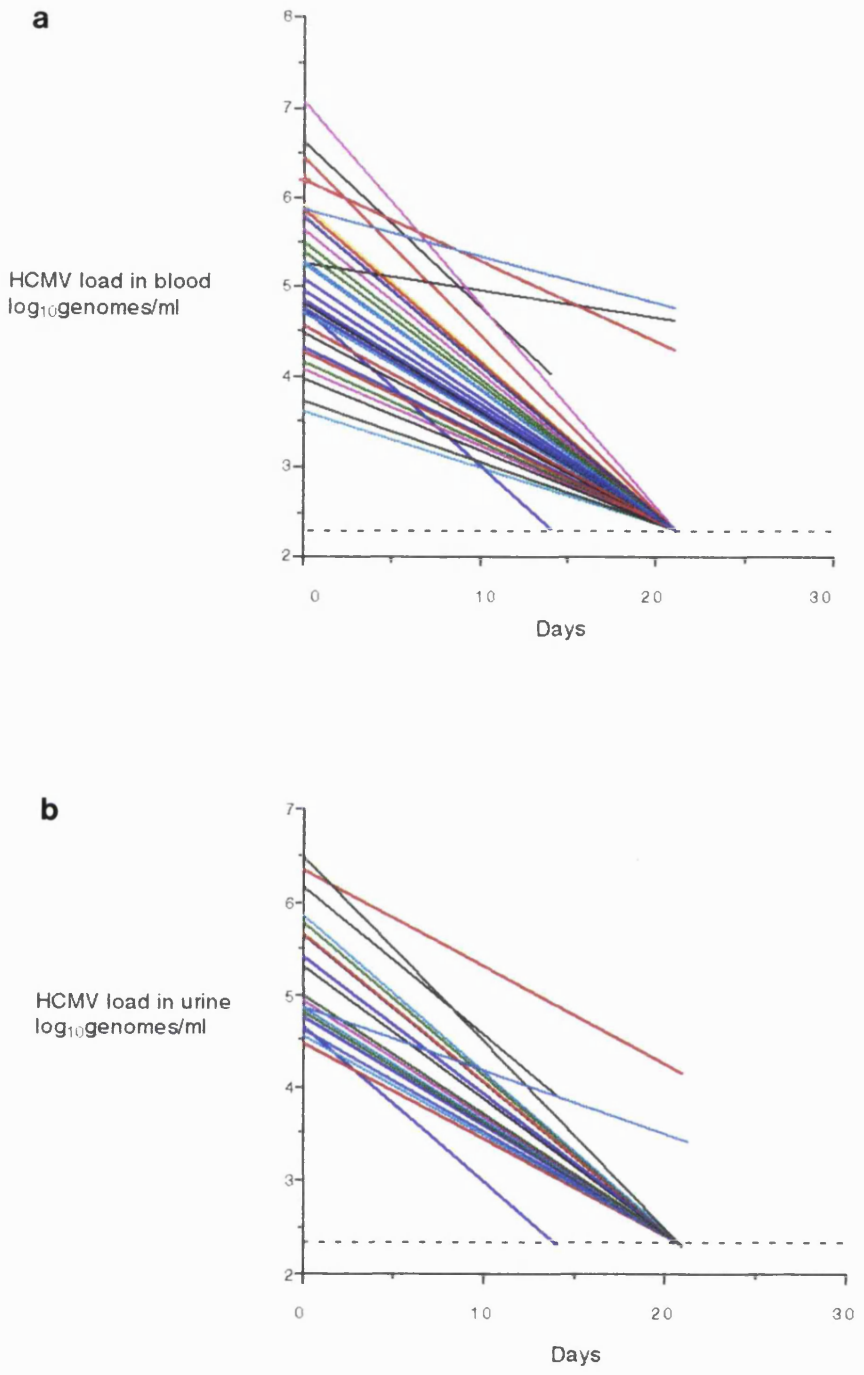

Figure 2.4: Reduction in HCMV load in blood (2.4a) and urine (2.4b) following ganciclovir induction therapy.

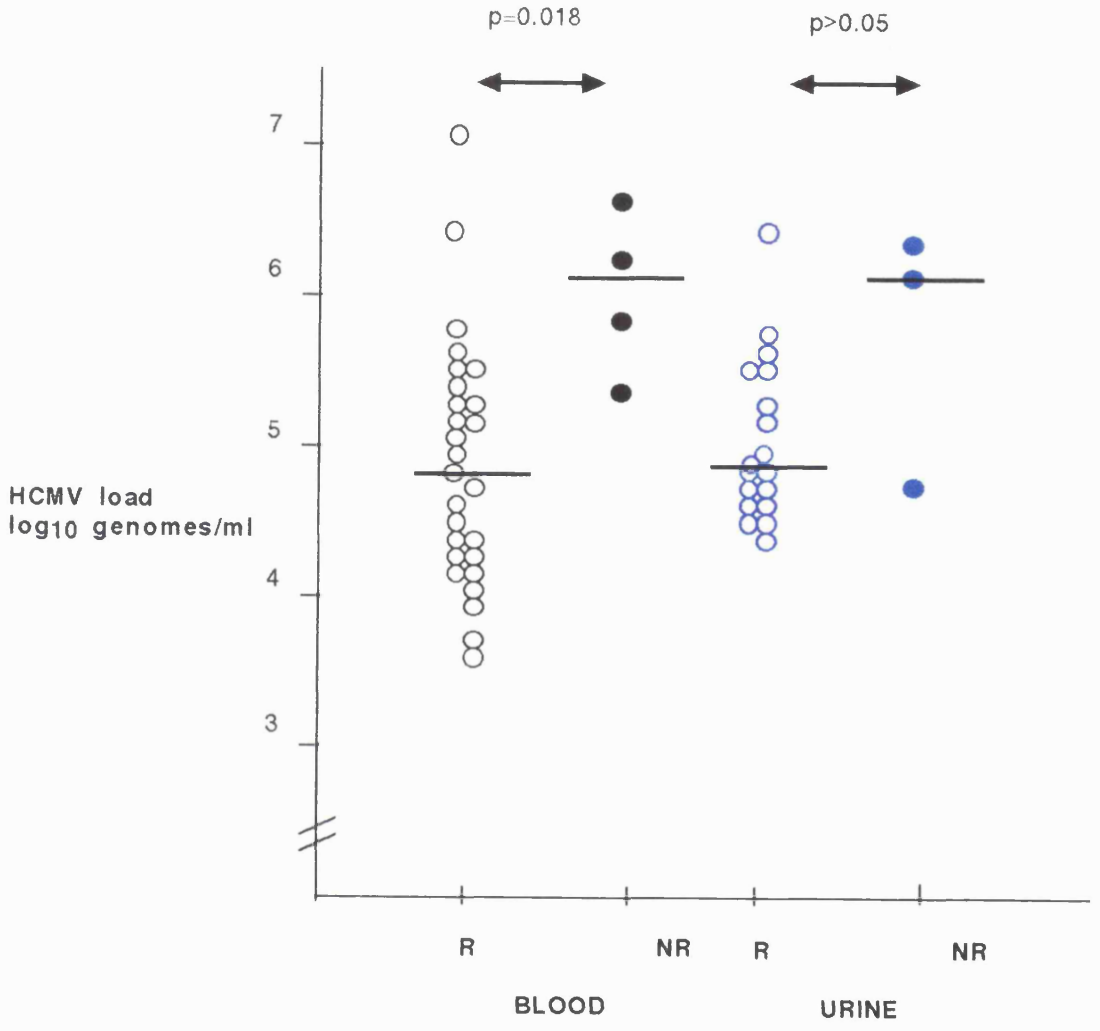

Figure 2.5: HCMV load at presentation of retinitis in blood and urine according to whether patients responded to ganciclovir induction therapy (R) or remained PCR positive (NR).

2.9.3 Relationship Between HCMV Load and Progression of Retinitis

Kaplan-Meier analysis showed no difference in the rates of retinitis progression between the 39 patients who were found to be PCR positive or the 6 who were PCR negative in blood at presentation (p=0.73, log rank test). When the analysis was restricted to those patients who were PCR positive in blood, those with high viral loads (ie > 4.95 $\log_{10}$ genomes/ml) appeared to have a shorter time to first progression of retinitis than those with lower viral loads, although this difference did not reach statistical significance (p=0.16, log rank test; see Figure 2.6a). Unadjusted for other factors, the hazard of progression of retinitis was increased by 40% for each log increase in the blood viral load (see Table 2.2), although, again, this did not reach statistical significance. No significant differences were found in progression rates in those found to be PCR positive in urine or in relation to the viral load in urine at presentation. There was however, as with blood viral load, a raised hazard for progression in those with higher urine viral loads (Table 2.2). Progression to retinitis was not related to method of drug administration (Figure 2.6b, p=0.84), patient's age (p=0.96), or CD4 count (p = 0.30). Interestingly, adjusting for these variables in a multivariate analysis resulted in a strengthening of the relationship between viral load both in blood and urine and time to retinitis progression (see Table 2.2), suggesting that these variables may have masked a more pronounced effect of HCMV load on time to retinitis progression. Despite this, the confidence intervals for the adjusted relative hazards all traversed unity and therefore, the effects remain non-significant.

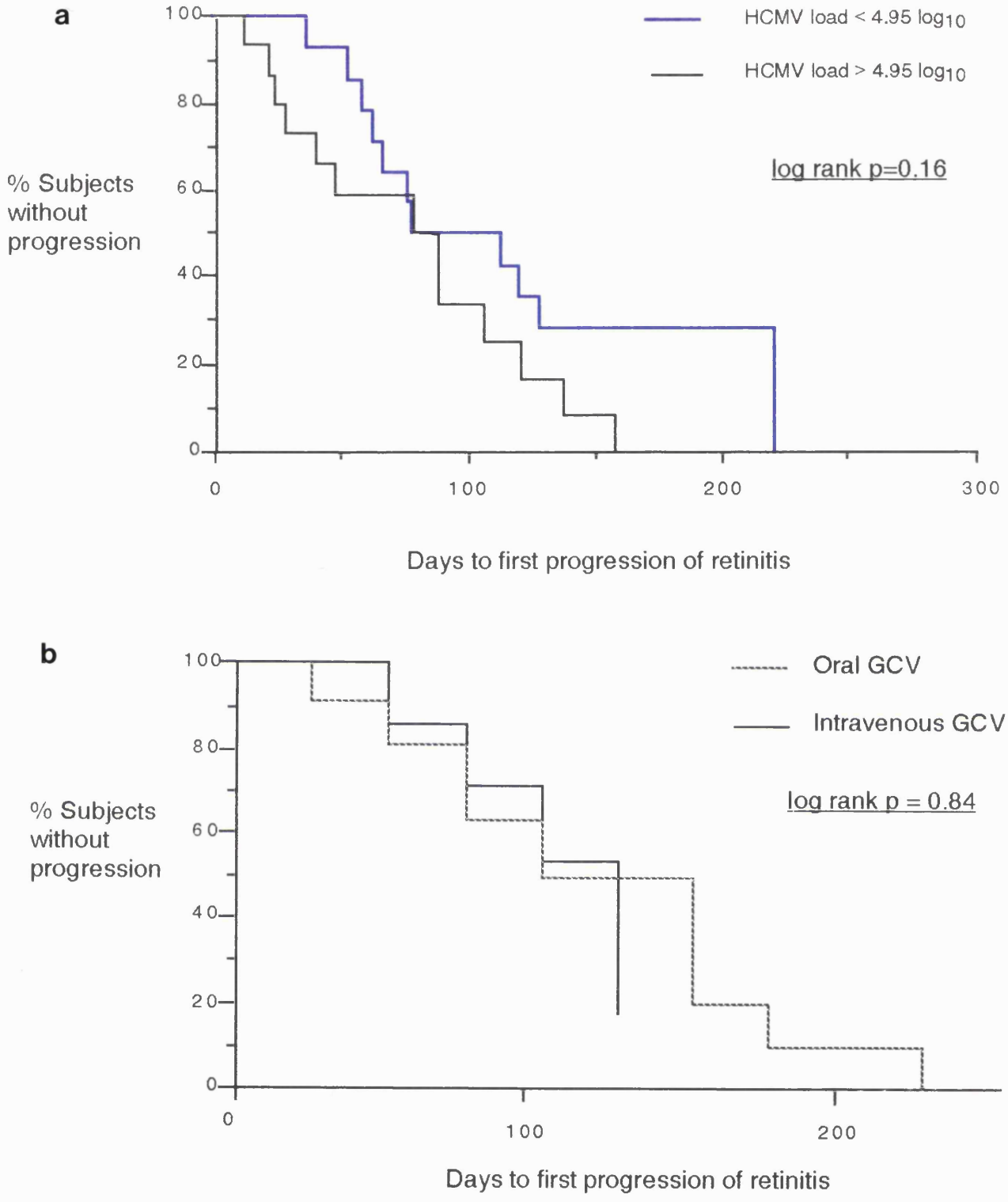

Figure 2.6: Kaplan-Meier analyses of time from initiation of maintenance therapy to first progression of retinitis according to (a) blood viral load at presentation and (b) mode of delivery of ganciclovir maintenance therapy, oral or intravenous (iv).

Table 2.2: Statistical analyses of the relationship between HCMV load in blood and urine and time to progression of retinitis or death.

	Time to first progression			Survival		
	Relative hazard **	**95% CI**	***p* value**	**Relative hazard ****	**95% CI**	***p* value**
↑Blood viral load:						
Unadjusted	1.40	0.46 - 2.31	$p = 0.18$	1.76	1.02 - 3.02	$p = 0.04$
Adjusted *	1.57	0.93 - 2.64	$p = 0.08$	1.88	1.05 - 3.35	$p = 0.03$
↑Urine viral load:						
Unadjusted	1.30	0.64 - 2.64	$p = 0.46$	1.76	0.53 - 5.83	$p = 0.36$
Adjusted *	1.83	0.78 - 4.29	$p = 0.17$	0.60	0.10 - 3.50	$p = 0.57$

*adjusted for age, CD4 count, and delivery of maintenance therapy
** for each 1 log increase in viral load

2.9.4 Relationship Between Cytomegalovirus Load and Survival

The association between viral load and survival was investigated using similar Kaplan-Meier analyses. There was no difference in survival between those who were PCR positive or negative in blood or urine (p=0.82 and p=0.57 respectively) at presentation. However, the Kaplan-Meier analysis for time to death revealed that an elevated viral load in blood at diagnosis of retinitis was associated with a significantly shorter time to death (log rank p=0.007; see Figure 2.7 a). The difference in median survival time between the two groups was 3.9 months (125 days). There was also a trend for elevated viral load in urine to be associated with a shorter survival but this did not reach statistical significance (p=0.10). Patients treated with intravenous ganciclovir also had a shorter time to death (p=0.02; Figure 2.7 b).

Unadjusted for other factors, the hazard of death was increased by 76% for each log increase in blood viral load and by 76% for each log increase in viral load in urine (see Table 2.2), although the latter did not achieve statistical significance. Intravenous administration of ganciclovir as maintenance treatment was associated with a 162% increase in the hazard of death (relative hazard 2.62, 95% CI 1.13 to 6.07, p=0.02). Neither age (p=0.75) nor CD4 count (p=0.44) were significantly associated with survival.

After adjusting for method of maintenance therapy administration, patient age and CD4 count in multivariate models, the risk of death was increased by 88% for each log increase in blood viral load.

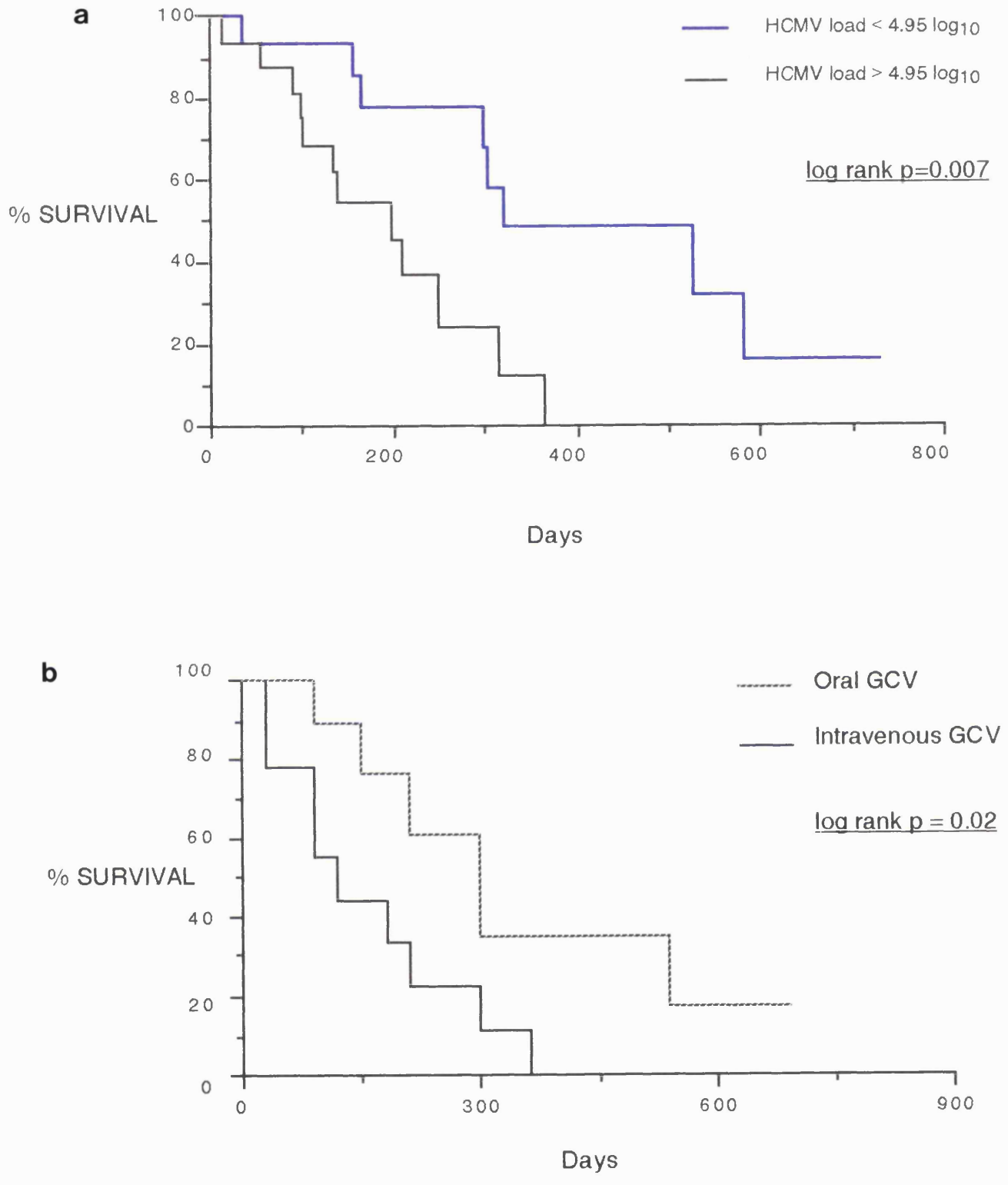

Figure 2.7: Kaplan-Meier analyses of time to death from the initial diagnosis of retinitis according to (a) blood viral load at presentation and (b) mode of delivery of ganciclovir maintenance therapy oral or intravenous (iv).

2.10 Discussion

This chapter describes the application of a quantitative-competitive PCR assay to investigate the importance of cytomegalovirus load in the pathogenesis of HCMV retinitis and to determine the effect of ganciclovir induction therapy on viral load. The results show that patients with a high HCMV load in blood at presentation of retinitis were less likely to become PCR negative after induction therapy, even in the presence of a good ophthalmological response. There was a trend for high HCMV loads in blood to be associated with a shorter time to retinitis progression, although this did not reach statistical significance. However, high viral loads in blood were associated with a significantly shorter time to death. In the majority of patients prescribed ganciclovir induction therapy there was a mean reduction of viral load in blood and urine of 2.8 $\log_{10}$ and 2.9 $\log_0$ genomes/ml respectively. Nevertheless, 6 patients who had significantly higher loads in blood at presentation remained PCR positive following induction therapy and had a median time to progression of 40 days compared to 78 days for the remaining patients who were PCR negative after induction therapy. This finding is consistent with data from Gerna *et al* who found that 15 (27%) of their 56 patients with retinitis remained HCMV DNA-positive in blood after 21 days of foscarnet induction therapy (Gerna *et al.* 1994a). These patients had a viral load at presentation significantly higher than those who had responded virologically to induction therapy.

The length of course of induction therapy is rather empirical as no controlled trials to ascertain the optimal length of treatment have been performed. From this study it appears that patients with low viral loads at presentation of retinitis may require shorter courses of induction therapy (ie 14 days) than that routinely used in the UK (21 days). This would greatly decrease the considerable discomfort and inconvenience to the patient as well as

treatment costs. However, as facilities for real time QCPCR are not widely available, I designed a study where those patients who become PCR negative in blood by day 10 could be randomized to receive 14 *vs* 21 days of induction therapy. Since January 1996 13 patients with HCMV retinitis have been recruited to such a study protocol. Two patients were still HCMV PCR positive at day 10, whilst one patient who had stable peripheral retinitis refused to be randomised in case he had to continue iv ganciclovir. To date, ten patients have been randomised, four to receive 21 days induction therapy and six to receive 14 days. Figure 2.8 shows the times to retinitis progression in each arm but the numbers were too small to reach statistical significance (log rank p= 0.14).

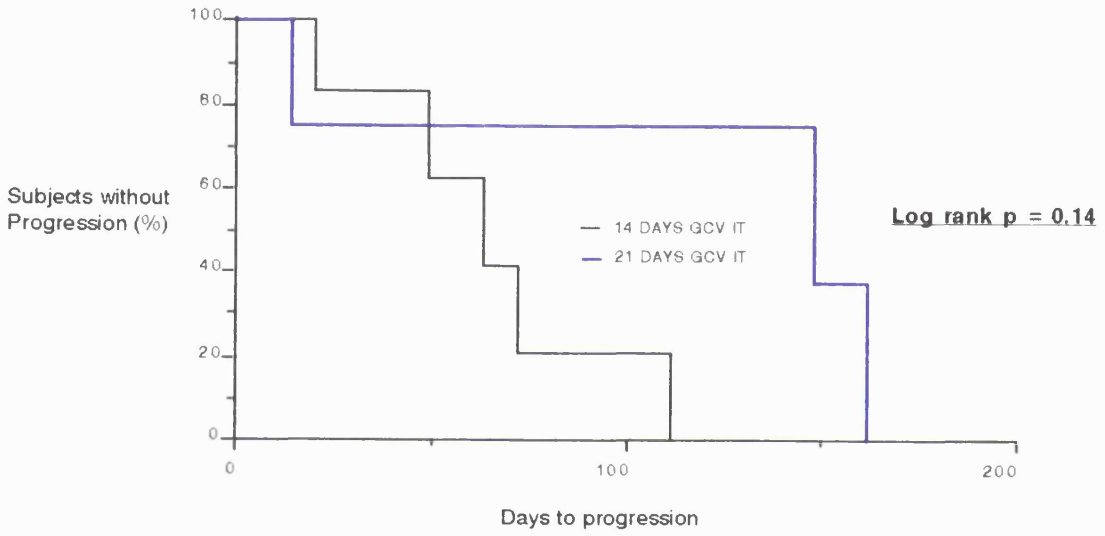

Figure 2.8: Kaplan-Meier analysis of time to first retinitis progression in patients HCMV PCR negative at day 10 of induction therapy (IT) who were randomised to receive either 14 or 21 days ganciclovir IT.

Whilst it is likely that HCMV viraemia is required for virus to seed the retina, 6 patients (13%) in this study had no evidence of HCMV by PCR at the time when they presented with retinitis. Two previous studies have reported similar rates of HCMV PCR negativity in patients with retinitis (16% and 14%, (Rasmussen *et al.* 1995; Gerna *et al.* 1994a) respectively). Hansen *et al* found that 5 out of 19 (26%) patients with HCMV retinitis were HCMV PCR negative prior to and at diagnosis of retinitis and remained so throughout their 9 months follow-up (Hansen *et al.* 1994). None of the 6 PCR negative patients in this thesis had samples taken before their retinitis was diagnosed so it is difficult to ascertain whether they had already had an episode of viraemia leading to the seeding of the retina or whether HCMV infection remained solely in the eye, thus acting as a privileged site. All six patients had unilateral disease at presentation. Five of these patients remained PCR negative during maintenance therapy despite local unilateral reactivations of their retinitis. The other patient remained PCR negative for many months before becoming PCR positive which was associated with fellow eye disease. Although this is a very small sub-cohort to draw conclusions from, HCMV retinitis appeared to behave in a more local, indolent fashion in these patients. Therefore, the treatment of choice in persistently HCMV PCR negative patients may be local therapy with either intra-ocular injections or ganciclovir implants.

In my study there was a non-significant ($p = 0.18$) trend for patients with higher baseline HCMV loads in blood to have a shorter time to progression. This effect was even more marked ($p = 0.08$) when calculated in a multivariate model adjusting for age, CD4 count and mode of delivery of maintenance therapy. This suggests that some of the above factors may be masking a more pronounced effect of HCMV load on retinitis progression. Certainly drug delivery/absorption and compliance with oral medication may be significant in retinitis

progression. In a retrospective analysis of 29 patients receiving foscarnet for HCMV retinitis the AUC produced with equivalent doses of foscarnet had a wide inter-individual range and, as such, the AUC of foscarnet significantly altered time to progression of retinitis (Drusano *et al.* 1996). This study also found that the only other significant factor for retinitis progression was baseline HCMV blood culture positivity where time to retinitis progression in HCMV culture positive patients was approximately 65 days compared to 180 days for HCMV culture negative patients. Assuming that HCMV culture is a surrogate marker for HCMV load and that only patients with high HCMV loads were culture positive, the quantity of HCMV in the blood at diagnosis of HCMV retinitis may have still have some bearing on time to retinitis progression.

There are a number of hypotheses which could account for the association between HCMV load and survival. For example, HCMV load may simply be a surrogate marker for a more profound level of immune dysfunction unrelated to the CD4 count. However, in a sub-group of 24 patients, in whom a stored serum sample from the time of diagnosis of their retinitis was available, p24 antigenaemia was measured. The relationship between HCMV load in the blood and decreased survival remained significant even after controlling for levels of p24 antigenaemia as either a continuous or categorical variable. Alternatively, since HCMV is found at multiple sites at autopsy (Pillay *et al.* 1993; D'Arminio Monforte *et al.* 1992) a high antemortem HCMV load may be a marker for a more widespread and more profound seeding of HCMV to other organs. This would imply that HCMV itself may be directly pathogenic and contribute to these early deaths; a possibility that could be further evaluated by monitoring survival in controlled trials of anti-HCMV therapy in AIDS patients. Indeed, there was a trend to an increased survival associated with oral ganciclovir "primary

prophylaxis" (relative hazard 0.82, (Spector *et al.* 1996) and 0.81, (Brosgart *et al.* 1992)) compared to placebo in patients with advanced HIV infection (CD4 <50 cells/μl) or an AIDS diagnosis. To ensure that the relationship between high viral load and decreased survival was not confounded by differences in ganciclovir maintenance therapy patient survival was analysed according to treatment received. Surprisingly, a strong association was seen in favour of oral ganciclovir maintenance therapy. Although there was a greater incidence of neutropaenia and anaemia in the group who received intravenous ganciclovir, there were no apparent differences in the number of bacterial septicaemias or line-related infections between the two groups. All 13 patients who received intravenous ganciclovir were recruited in 1993 and all 13 had a previous AIDS diagnosis compared to 82% of those receiving oral ganciclovir (6 patients had HCMV retinitis as their AIDS-defining illness). This suggests that the survival difference between the two groups may reflect improvement in patient prognosis with time (Hoover *et al.* 1993). No survival difference was observed in a large randomised trial of oral *vs* intravenous ganciclovir where the majority of patients were receiving combination anti-retroviral therapy (Drew *et al.* 1995). Since the patients in my study were not randomised to treatment, further study would only be warranted if a similar effect was seen from follow-up studies of patients randomized to treatment arms in controlled trials. Certainly the differences in maintenance therapy do not confound the association between HCMV load and survival because HCMV load remained significantly associated with survival after adjustments for maintenance therapy in a multivariate analysis (Table 2.2).

CHAPTER 3

A PROSPECTIVE STUDY OF HCMV PCR VIRAEMIA IN AIDS PATIENTS RECEIVING MAINTENANCE THERAPY FOR HCMV RETINITIS

3.1 Introduction

There is a paucity of data on the use of molecular methods to monitor AIDS patients with HCMV retinitis for the recrudescence of their retinitis or the development of other HCMV related disease. In a study in 1990 using conventional cell culture for HCMV in 14 patients on ganciclovir (GCV) maintenance therapy for HCMV retinitis, 41% of patients on low dose GCV maintenance therapy (5 mg/kg for 5 days per week) had HCMV isolated compared to only 16% on a higher dose (5 mg/kg for 7 days per week) (Jennens *et al.* 1990). There was a significant correlation between the stability of retinitis and the prevalence of HCMV viraemia, irrespective of dose regimen, with only 10% of those patients with stable retinitis having HCMV isolated compared to 61% of patients with progressive disease. In a smaller study, 35 assays for pp65 antigenaemia were performed on 11 patients who received GCV induction and maintenance therapy (Salzberger *et al.* 1996). In these patients there was a significant correlation between therapeutic outcome and negative antigenaemia during maintenance therapy. Six patients, who became pp65 antigenaemia negative following GCV induction therapy, became positive again on maintenance therapy coinciding with recurrent disease. The presence of pp65 antigenaemia was also found to coincide with confirmed disease progression in another small study where 10 out of 13 (76%) patients with a spectrum of HCMV diseases had progression of their disease (Bek *et al.* 1996). However, in neither of these studies was a time course given to illustrate the correlation between the recurrence of antigenaemia and progression of retinitis/HCMV related disease.

Only two small studies have prospectively investigated the relationship between HCMV PCR and HCMV disease progression in AIDS patients. Hansen *et al* followed 19 patients with HCMV retinitis of whom 14 were PCR positive at diagnosis of retinitis. Samples were

collected on a three monthly basis after diagnosis (Hansen *et al.* 1994). Four patients remained PCR positive throughout the study. Of the 10 patients who became PCR negative following induction therapy, 4 became PCR-positive on maintenance therapy and 6 remained PCR negative. The significance of these findings in relation to disease progression were not discussed. Drouet *et al* in 1993 studied the natural history of eight patients who developed HCMV retinitis (out of 110 HIV positive patients under longitudinal follow-up), 6 of whom continued to be followed whilst receiving maintenance therapy (Drouet *et al.* 1993). All 6 patients were PCR negative after 2-4 weeks of induction therapy. Four patients became PCR positive with their first progression of retinitis. The other 2 patients were PCR negative at progression of retinitis but subsequently became PCR positive with a "relapse" of other HCMV related symptoms/disease. This small study implied that there was a correlation between HCMV PCR positivity and HCMV disease progression which needs to be further evaluated.

This chapter describes the prospective follow-up of 45 AIDS patients receiving maintenance therapy for HCMV retinitis. Monthly HCMV PCR was performed on blood and urine samples in conjunction with monthly ophthalmological and medical follow-up in order to correlate PCR results with clinical outcome. Wherever possible (with the patients' and partners' consent), multiple tissue samples were taken at post-mortem to study the distribution of HCMV throughout the body for comparison with ante-mortem clinical symptoms.

3.2 Methods

3.2.1 Patients

Forty-five HIV positive patients were prospectively recruited to this study following a diagnosis of HCMV retinitis (cohort fully described in section 2.2) and were followed on a monthly basis whilst on maintenance therapy with ophthalmological, medical and virological follow-up. Patients were usually seen by the same ophthalmologist at each monthly visit. Any patient who noticed a new deterioration in vision or new visual symptoms was seen in the eye clinic as soon as possible. If retinitis had progressed, extra samples of blood and urine were collected at that time. In addition, aliquots of other samples, such as vitreous fluid or CSF taken for diagnostic purposes, were collected for HCMV analysis. In cases where either the patient, partner or next of kin had given written consent for a post-mortem, multiple tissue samples were taken at autopsy. Apart from the AIDS patients with HCMV retinitis, tissues taken from other AIDS patients at post-mortem were also studied in comparison to control patients who were believed not to be seropositive for HIV infection. Maintenance therapy was given as described in section 2.2.3

3.2.2 DNA Extraction From Clinical Samples

DNA was extracted from whole blood and vitreous fluid as detailed in section 2.3. Urine and CSF were analysed without extraction.

3.2.3 Extraction of DNA From Post Mortem Tissues

Samples were taken at post-mortem (PM) from a standard list of different organs that was used when collecting PM samples; lymph node, spleen, brain, lung, heart, kidney, adrenal, oesophagus, duodenum, colon, pancreas, liver, stomach and salivary gland. The

samples were divided into two; one for cell culture and one for storage immediately at -70°C.

All DNA extractions from PM material were carried out in a separate Cass II cabinet where HCMV samples were not routinely processed. On thawing, a $1cm^3$ block was cut from each tissue, finely dissected and washed three times in 1ml PBS to remove residual blood. The samples were then washed three times in lysis buffer (10mM TRIS-HCl. pH8, 1mM EDTA and 0.5% SDS (w/v)) for two minutes and incubated overnight at 37°C in 1ml of extraction buffer (10mM TRIS-HCl, pH8, 10mM EDTA, 1% SDS (w/v) and 50mg/ml proteinase K). Following the fourth extraction, cellular DNA was extracted twice with the addition of an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) to the supernatant followed by microcentrifugation at 13,000g. The supernatant was removed and re-extracted with chloroform:isoamylalcohol (24:1), microcentrifuged and DNA precipitated from the supernatant with the addition of 1/10 volume of 3M sodium acetate and 2 volumes of 100% ethanol followed by incubation at -70°C for 1 hour. The DNA was pelleted by microcentrifugation at 13,000g for 20 min, washed with 200µl 70% ethanol, vacuum dried, resuspended in 200µl of TE and stored at -70°C.

The absorbance (A) of the DNA was measured at 260nm using a spectrophotometer and the DNA concentration calculated according to the equation: 1 A_{260} unit = 50µg/ml double stranded DNA. 1µg of extracted tissue DNA was used in each PCR reaction.

3.2.4 Qualitative and Quantitative PCR for HCMV

Qualitative PCR was performed exactly as described in section 2.4 All samples that were qualitatively positive for HCMV DNA were subjected to quantitative analysis using the quantitative-competitive PCR (QCPCR) method described in section 2.5.

When analysing DNA extracted from PM samples, 1µg of DNA was used in each QCPCR

reaction. However, in some instances the concentration of target HCMV DNA was so great (ie $>10^5$ copies/μg) that multiple dilutions of tissue DNA from 1 in 10 to 1 in 1000 had to be made.

3.3 Statistics

The significance of becoming HCMV PCR positive on maintenance therapy in relation to progression of retinitis and the development of other HCMV disease, was assessed using 2 x 2 tables and the χ^2 test for significance. Differences in time to first progression of HCMV retinitis according to HCMV PCR status on maintenance therapy were analysed using both Kaplan-Meier plots with the log-rank test for significance and the Mann-Whitney U test for the comparison between two groups. The latter test was also used to compare HCMV load in blood prior to and at diagnosis of retinitis progression.

3.4 Results

3.4.1 Patients

Forty five patients from the cohort described in section 2.2 received maintenance therapy. All 45 patients were initially treated with ganciclovir (either intravenously or orally) and median follow-up was 8.7 months (range 1-16 months). Thirty seven patients (82%) had at least one progression of their retinitis, the remainder having died without retinitis progression. Thirty four patients remained on ganciclovir maintenance therapy throughout (oral or iv). Six patients were changed to foscarnet (4 with progressive HCMV retinitis on ganciclovir, 1 with progressive disease and evidence of UL97 mutations in the peripheral blood, and 1 with stable retinitis but persistent pancytopenia refractory to GCSF treatment). The remaining five patients with progressive retinitis were enrolled into a randomised study of cidofovir 3mg/kg iv vs 5mg/kg iv given weekly for two weeks as induction therapy and fortnightly thereafter. Cidofovir was administered in an "infusion setting" in the day centre with strict patient monitoring, rigorous pre-hydration and the concomitant administration of oral probenecid to reduce potential nephrotoxicity.

3.4.2 Qualitative PCR Results

The median number of follow-up HCMV PCR's performed per patient was 7 (range 1 to 18). A total of 351 qualitative HCMV PCR measurements on whole blood and 312 on urine were performed during the follow-up period. Twenty patients (44%) were found to be PCR positive in blood and five (11%) in urine at some time whilst receiving maintenance therapy.

3.4.3 Correlation Between HCMV PCR and First Progression of Retinitis

At the time of first retinitis progression in the 37 patients who progressed, 11 (30%) were HCMV PCR positive in blood alone, 2 patients were PCR positive in blood and urine (5%) and 1 patient (3%) was PCR positive in urine alone. 23 patients were PCR negative (62%) at first progression of retinitis. There was a significant association between becoming HCMV PCR positive and having a progression of retinitis ($\chi^2 = 4.39$, p=0.036, Table 3.1). Although the majority of patients were PCR negative at the time of retinitis progression, HCMV PCR had a specificity of 100% for the progression of retinitis with a positive predictive value of 100%. However, the negative predictive value and sensitivity of HCMV PCR were 25% and 38% respectively suggesting that this assay cannot be used to replace routine ophthalmological follow-up.

Four patients became PCR negative following re-induction with high dose ganciclovir whilst the other ten patients remained PCR positive. This contrasts with patients receiving 'primary' induction therapy where 85% of patients became PCR negative as discussed in section 2.10.

3.4.4 Correlation Between HCMV PCR and other HCMV Disease

Becoming HCMV PCR positive at any time whilst on maintenance therapy was significantly associated with the development of other end-organ HCMV disease ($\chi^2 = 10.81$, p =0.001, Table 3.2). Nine patients who were HCMV PCR positive developed other systemic HCMV disease during follow-up; 3 cases of HCMV oesophageal ulceration, 2 cases of HCMV encephalitis and 4 cases of HCMV hypoadrenalism (diagnosed by an abnormal response to a short synacthen test followed by symptomatic improvement on gluco- and mineralo-corticosteroid replacement therapy). One patient developed HCMV

polyradiculopathy and was HCMV PCR positive in CSF but PCR negative in both blood and urine. In addition, one patient, who was persistently PCR positive on maintenance therapy, developed and subsequently died from acute pancreatitis (he was taking neither ddI nor ddC). HCMV is reported to cause pancreatitis but as this patient did not have a post mortem this event is not included in the 'other' HCMV diseases.

3.4.5 Time to First Progression and HCMV PCR Status

In those patients who had an episode of retinitis progression, the time to first progression of retinitis was significantly shorter in patients who were PCR negative at the time of their first progression (median time 61 days vs 105 days if PCR positive; Mann-Whitney U test, $p = 0.04$). However, if Kaplan-Meier survival analysis was used to assess time to first progression, taking into account the 8 PCR negative patients whose retinitis did not progress, there was no significant difference in time to first progression according to HCMV PCR status (log-rank test $p = 0.24$, see Figure 3.1).

Table 3.1: Relationship between HCMV PCR positivity and first progression of HCMV retinitis.

	Retinitis progression		
HCMV PCR status at first progression	**Yes**	**No**	**Totals**
Positive	14	0	14
Negative	23	8	31
Totals	37	8	45

$\chi^2 = 4.39$, p=0.036.

Table 3.2: Relationship between PCR positivity and the development of other HCMV related systemic disease.

	HCMV disease		
HCMV PCR status on maintenance therapy	**Yes**	**No**	**Totals**
Positive	9.	11	20
Negative	1	24	25
Totals	10	35	45

$\chi^2 = 10.81$, p =0.001

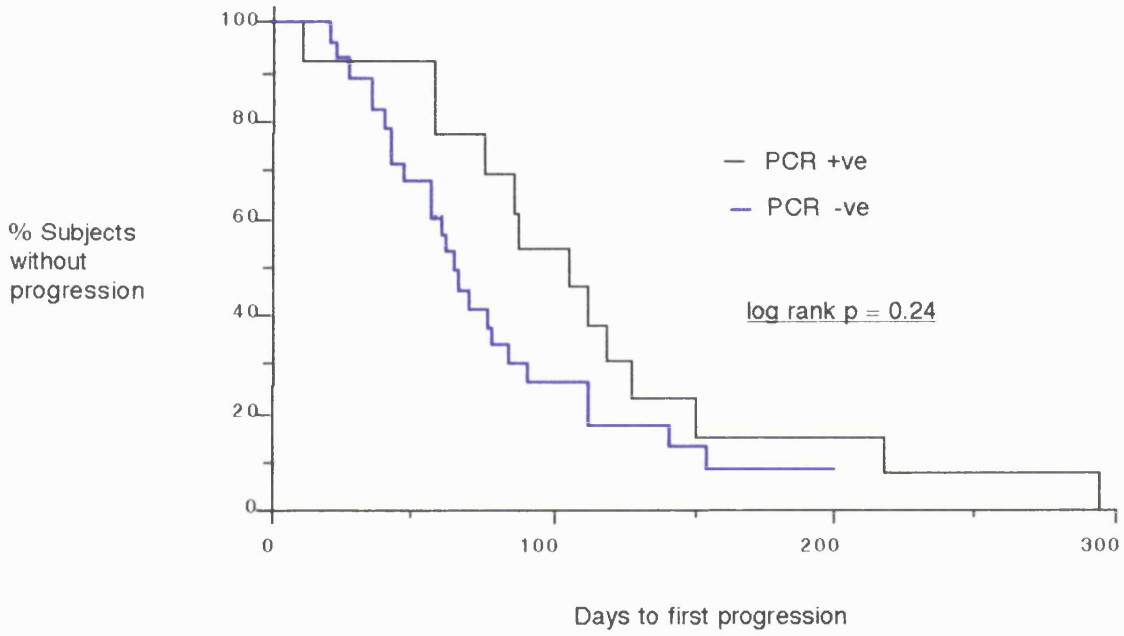

Figure 3.1: Kaplan-Meier analysis of time to first progression of retinitis according to HCMV PCR status at time of first progression.

3.5 Quantitative PCR Results

Seven of the thirteen patients described in section 3.4.3 who were HCMV PCR positive in blood at the first progression of their retinitis were HCMV PCR positive prior to their retinitis progression (Figure 3.2). At initial PCR positivity, median HCMV load was 4.4 $\log_{10}$ genomes/ml blood compared to 5.51 $\log_{10}$ genomes/ml at progression of retinitis (Mann Whitney U test $p < 0.05$). There was no correlation between each patients HCMV load at their initial presentation with retinitis and at the time of retinitis progression. In the patients who were HCMV PCR positive prior to documented retinitis progression, there was no evidence of active retinal lesions when reviewed in the ophthalmology department either at their routine appointment or at an extra appointment arranged due to their PCR viraemia. In one patient (see Figure 3.2 and RM in Figure 3.3) HCMV load peaked prior to progression and was undetectable at the time of retinitis progression.

The relationship between HCMV load, retinitis progression, drug therapy and clinical outcome is shown for some of the patients who became HCMV PCR positive during follow-up in Figures 3.3 to 3.5. Figure 3.3 shows two patients (MT and RM) who were managed on ganciclovir therapy alone for their retinitis progression, and TE who was changed to foscarnet in an attempt to optimise the control of his retinitis. Figures 3.4 and 3.5 show four patients who were enrolled into the randomised trial of 3 vs 5mg/kg of cidofovir for progressive HCMV retinitis. These four patients were generally found to have a good virological response to ganciclovir or foscarnet re-induction therapy but all became, or remained, HCMV PCR positive on cidofovir. The correlation between remaining PCR positive and the development of other HCMV related disease is also shown in Figures 3.3 to 3.5.

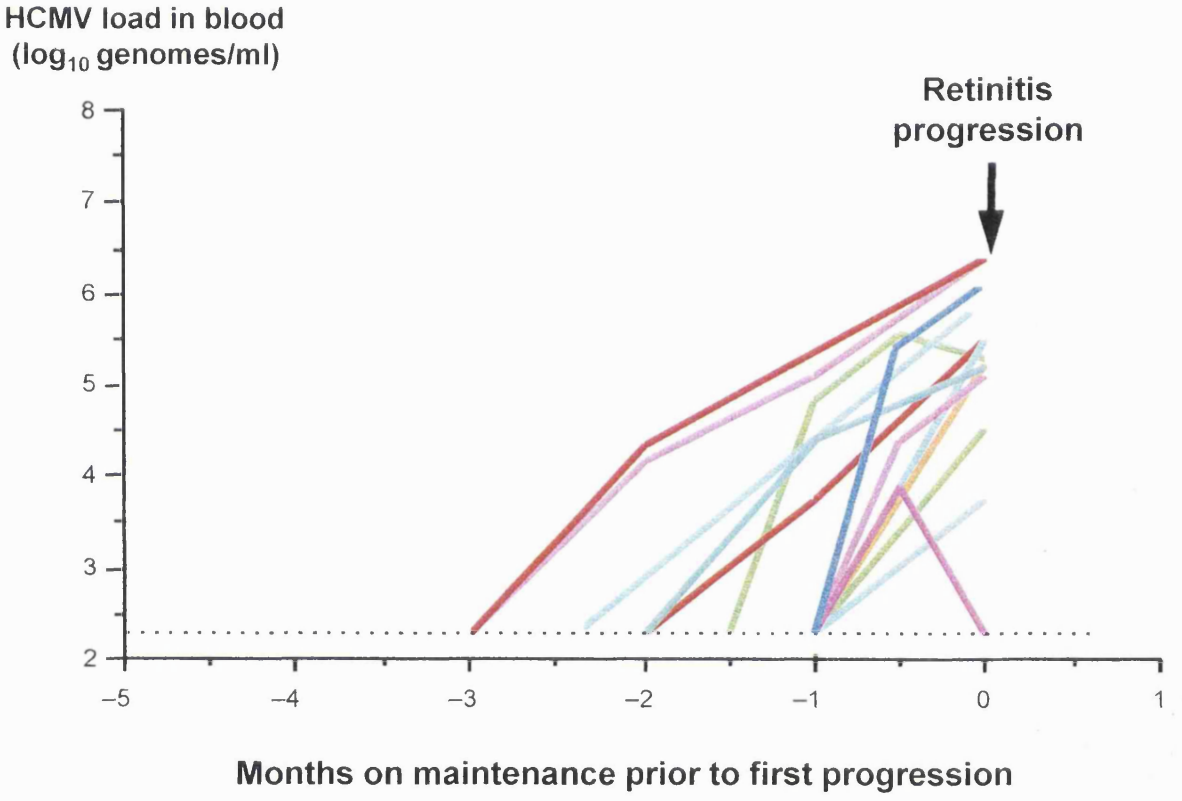

Figure 3.2: Changes in HCMV load in blood during maintenance therapy prior to the first progression of retinitis in all patients who were HCMV PCR positive at their first progression.

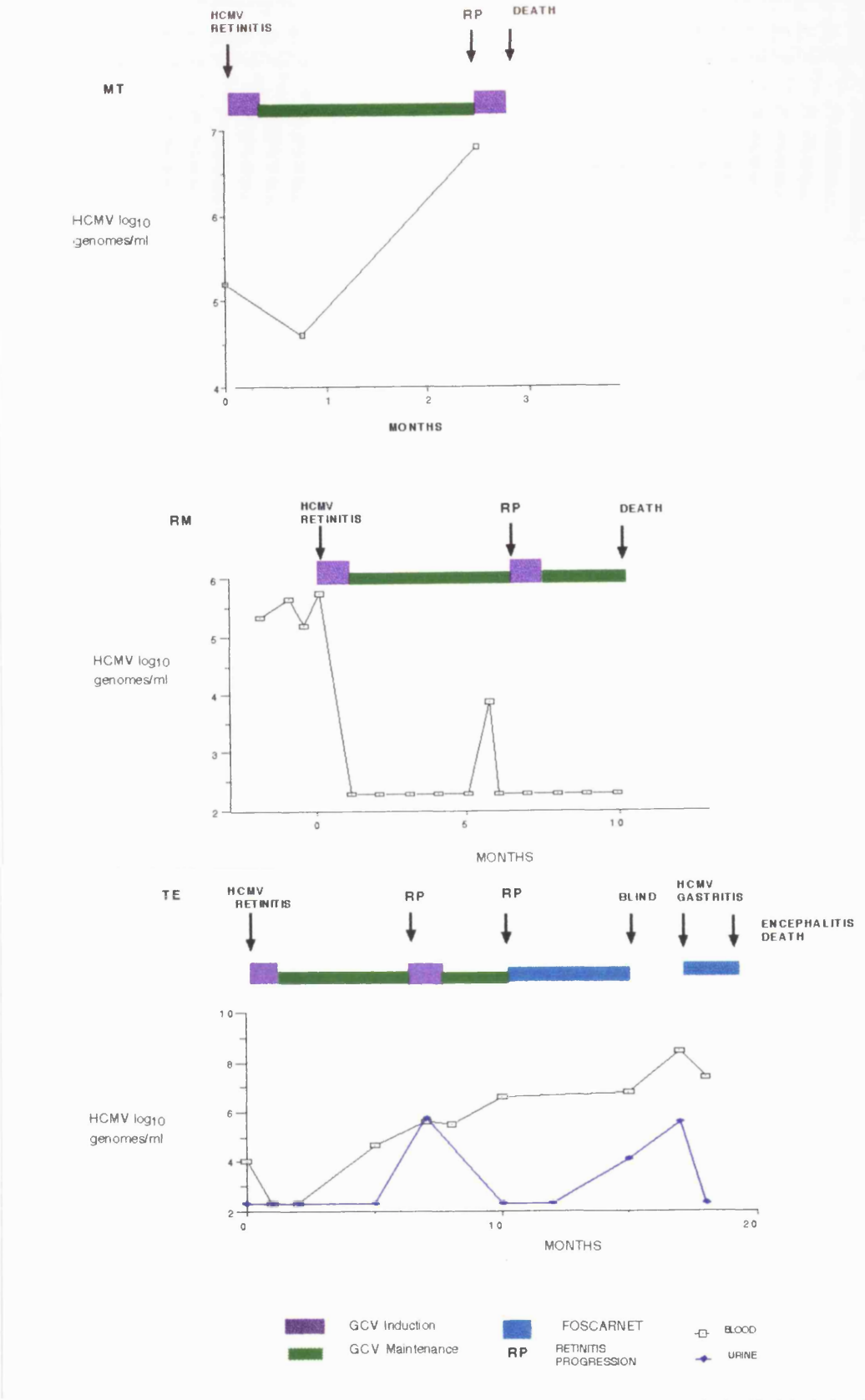

Figure 3.3: Changes in HCMV load in individual patients during maintenance therapy in relation to retinitis progression, other HCMV disease and anti-HCMV therapy.

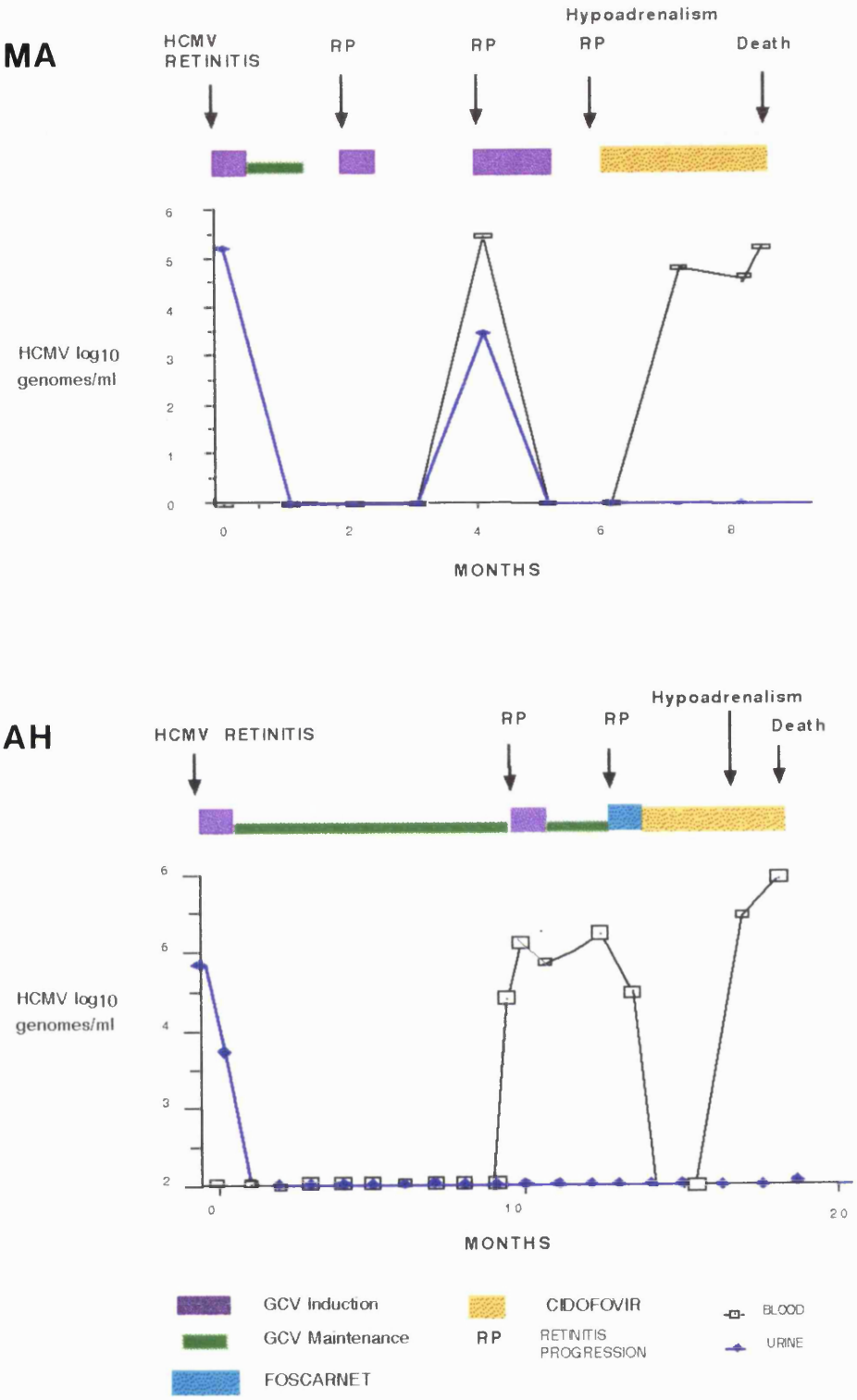

Figure 3.4: Changes in HCMV load in individual patients during maintenance therapy in relation to retinitis, other HCMV disease and anti-HCMV therapy, including cidofovir.

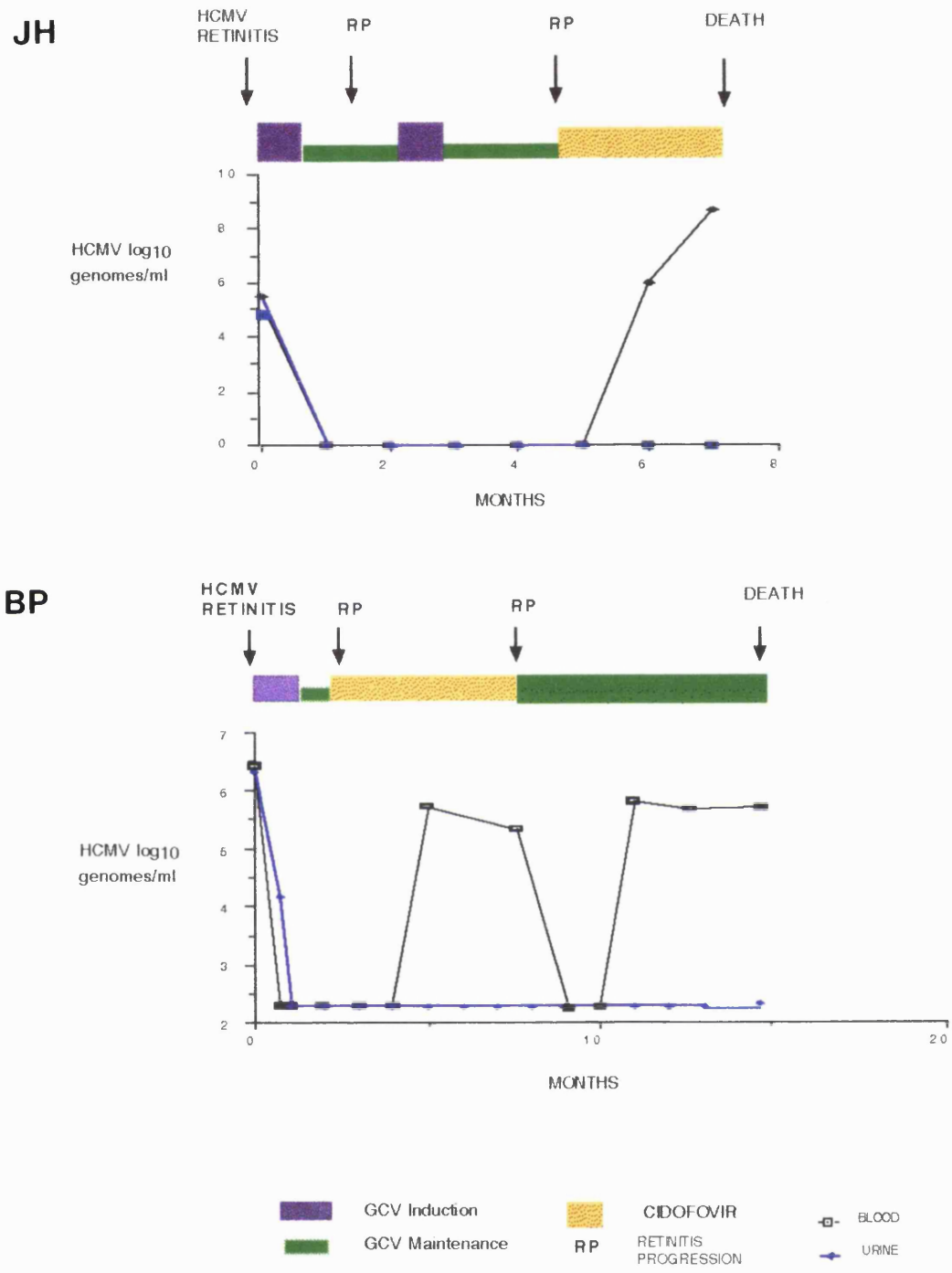

Figure 3.5: Changes in HCMV load in individual patients during maintenance therapy in relation to retinitis, other HCMV disease and anti-HCMV therapy, including cidofovir.

3.6 HCMV Distribution in Post Mortem Samples

Fifteen patients had multiple tissue samples taken at post mortem for HCMV PCR analysis. They included two patients with HCMV retinitis from this cohort, one patient with progressive HCMV polyradiculitis and nine other patients with AIDS who, as part of an ongoing departmental study investigating the prevalence of herpesviruses in AIDS patients, had post mortem tissue samples stored. Three patients, all young men who died suddenly and were HIV negative were used as negative controls. All samples were initially analysed qualitatively for HCMV gB as described in section 2.4 and positive samples were subsequently subjected to quantitative analysis by the QCPCR described in section 2.5. The distribution and quantity of HCMV in each tissue for every patient is shown in Table 3.3.

There was a marked difference in the frequency of HCMV found in the control patients and the patients with AIDS. The only tissues with HCMV DNA in the control patients were the spleen and lung in all three patients, the liver in one patient and the salivary gland in two. In contrast, HCMV was widely distributed throughout most of the tissues of the AIDS patients. HCMV DNA was detected in the adrenal glands of all the AIDS patients and was detected in the lung, spleen and lymph nodes in the majority of patients.

The median HCMV load for all tissues processed was significantly higher in the AIDS patients than the control patients (2.88 log vs 0.6 $\log_{10}$ genomes/μg DNA, $p<0.0001$) as was the median HCMV load for each individual tissue (Table 3.3), however due to the small number of control patients, this was not significant.

Table 3.3: The distribution and quantity of HCMV in each tissue for every patient.

Patient	L node	Spleen	Brain	Lung	Heart	Kidney	Adrenal	Oesoph.	Duod.	Colon	Pancreas	Liver	Stomach	Salivary gland
Control 1	-	2.2	-	1.5	-	-	-	-	-	-	-	-	-	-
Control 2	-	3.89	-	2.29	-	-	-	-	-	-	-	2.39	-	3.86
Control 3	-	2.16	-	3.27	-	-	-	2.71	-	-	-	-	-	2.67
MEDIAN		**2.2**		**2.29**				**2.71**				**2.39**		**3.2**
DW	2.69	2.98	-	3.73	-	4.41	**7.96**	3.46	**5.13**	2.4	-	1.76	-	2.75
AV	5.57	NA	**5.29**	NA	-	-	**5.53**	4.28	2.79	4.23	-	-	3.18	3.82
JH	**6.42**	NA	**4.71**	**8.43**	-	NA	**8.05**	**6.42**	**5.49**	NA	NA	NA	NA	**5.21**
FK	-	-	4.56	-	-	-	NA	NA	NA	NA	NA	-	NA	-
DH	2.49	-	NA	2.01	-	-	2.99	-	-	-	NA	-	-	-
JC	3.9	3.57	3.06	3.5	NA	3.13	4.79	3.54	2.87	2.62	-	3.08	-	-
PH	5.66	4.93	4.82	5.34	4.88	5.17	**6.84**	NA	NA	NA	NA	5.23	NA	4.93
SS	-	-	-	-	-	-	3.53	2.29	-	3.05	-	-	2.42	-
BP	-	2.0	-	5.27	-	-	2.88	1.6	4.01	3.14	-	-	-	-
EW	4.29	5.25	**6.23**	**7.28**	**5.65**	3.68	**6.99**	4.38	NA	NA	4.83	5.36	5.08	5.11
HT	4.12	4.54	2.88	NA	3.85	-	**4.36**	**5.0**	4.01	4.93	3.14	NA	NA	3.78
JHenn.	2.7	-	3.2	2.42	-	-	3.76	2.48	-	-	-	-	-	2.94
MEDIAN	**4.12**	**4.05**	**4.6**	**4.5**	**4.7**	**4.0**	**5.2**	**3.5**	**4.0**	**3.0**	**3.9**	**4.1**	**3.18**	**3.8**

All values in $\log_{10}$ genomes/μg DNA, apart from vitreous in $\log_{10}$ genomes/μl vitreous fluid. NA = tissue not available; - = HCMV undetectable by PCR. Shaded squares = HCMV demonstrated at histological examination.

3.6.1 Correlation Between HCMV Load and Histological Findings

As can be seen from Table 3.3 there was a correlation between tissue samples found to have the highest HCMV loads and evidence of HCMV infection diagnosed histologically (indicated by the shaded squares). Conversely, there were no instances where HCMV was detected histologically and was not found by PCR ($\chi^2 = 13.42$, p=0.0002, see Table 3.4). The median HCMV load was significantly higher in the tissues where HCMV had also been detected histologically compared to the tissues where there was no evidence of HCMV (5.94 log vs 3.57 log respectively, Mann Whitney U test, $p < 0.0001$, Figure 3.6).

There were five patients who had an ante-mortem diagnosis of HCMV disease confirmed (DW, retinitis; AV, polyradiculopathy and encephalopathy; JH, retinitis and adrenalitis; EW, retinitis and JC, retinitis). All five had HCMV detected both by PCR and by histology in various organs. Interestingly, JC who had been on ganciclovir therapy for over 28 months for his retinitis, had no evidence of HCMV disease histologically and the HCMV loads in each tissue were lower than many of the other patients.

Table 3.4; Relationship between HCMV PCR organ positivity and the histological demonstration of HCMV infection.

HCMV PCR of PM tissue	**HCMV histologically YES**	**HCMV histologically NO**	**Totals**
Positive	18	68	86
Negative	0	56	56
Totals	18	124	142

$\chi^2 = 13.42$, $p=0.0002$

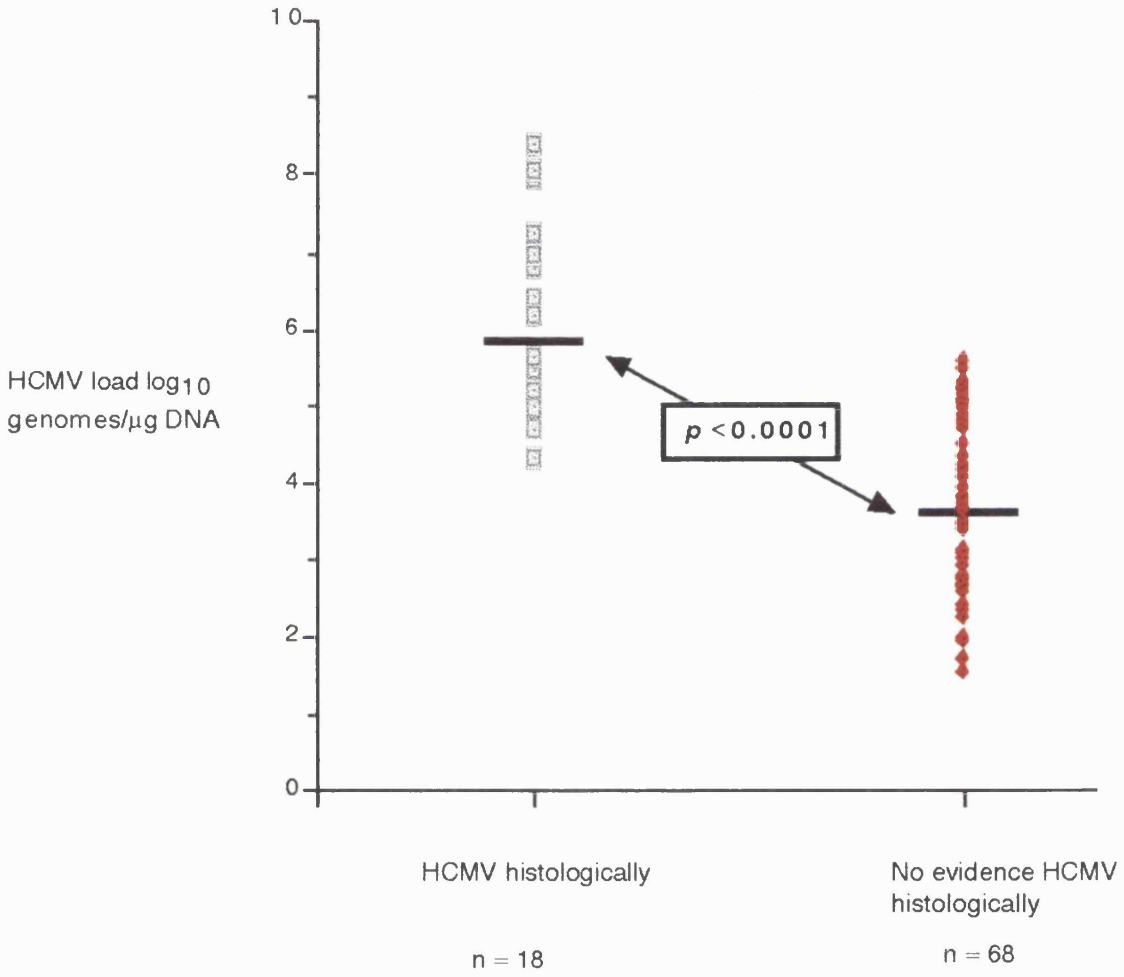

Figure 3.6: Median HCMV loads in the AIDS patients according to whether HCMV was demonstrated at histological examination or not.

3.7 Discussion

The aim of this chapter was to provide insight into the natural history of HCMV viraemia, as detected by PCR, in patients with HCMV retinitis receiving maintenance therapy and to evaluate if PCR could be used to monitor maintenance therapy in a similar way to monitoring induction therapy as described in section 2.10. Over 85% of patients with retinitis, both in this and other studies, become PCR negative following high dose induction therapy and therefore, are PCR negative when they commence maintenance therapy (Gerna *et al.* 1994; Hansen *et al.* 1994). Forty four percent of patients in this study became HCMV PCR positive at some time during follow-up which is lower than the 55% and 76% of HCMV pp65 positive patients in the smaller studies of Salzberger and Bek respectively (Salzberger *et al.* 1996; Bek *et al.* 1996). It is difficult to compare the results of these studies with the results described in this chapter as two different assays have been employed to detect HCMV in three different patient groups.

The majority of patients (65%) I studied were found to have a recurrence of their retinitis without any evidence of systemic HCMV replication. Again, this is lower than the HCMV positive recurrences observed in the studies mentioned above, although the maintenance regimens used in those studies were not detailed. The most likely reason for retinitis progression in the absence of HCMV viraemia is the local reactivation of retinal disease in the presence of sub-optimal vitreal concentrations of anti-viral chemotherapy. This implies that regular ophthalmological follow-up remains the most sensitive method for detecting progression of retinitis. However, an HCMV PCR positive result does have important implications as all of the 14 patients who became HCMV PCR positive in blood had a progression of their retinitis and were more likely to develop HCMV disease elsewhere.

There was a trend for a longer time to retinitis progression in patients who were PCR positive. One explanation for this could be that retinitis progression in this sub-set of patients is due to reinfection of the retina rather than local reactivation. This interpretation is supported by the fact that 50% of the patients had been PCR positive for approximately one month prior to progression and HCMV load increased prior to progression. Therefore, in these patients, particularly if they are receiving oral ganciclovir maintenance therapy, a "pre-emptive" course of high dose re-induction therapy might delay retinitis progression. Obviously the potential benefits of re-induction to prevent progression must be weighed against the possible complications surrounding the use of intravenous catheters and high dose ganciclovir.

Unlike patients receiving ganciclovir induction therapy, the majority of patients who were HCMV PCR positive when their retinitis progressed, did not become PCR negative after 14 days of high dose GCV therapy. This may be due to the presence of HCMV strains carrying mutations in UL97 which may give rise to phenotypic GCV resistance and this explanation is explored in more detail in Chapter 4 of this thesis. In the five patients in this study who received cidofovir (CDV), two remained PCR positive with virtually no reduction in blood HCMV load following the introduction of CDV, while the other three patients became PCR positive on CDV therapy. Despite the persistently high levels of HCMV viraemia in these five patients, all five had immediate stabilisation of their retinitis with no further episodes of progression. However, two of these patients developed hypoadrenalism and one patient developed and died from acute pancreatitis. It appears therefore, that CDV may be concentrated in the retina allowing local control of retinitis but with little benefit on blood borne virus, which ultimately seeds other organs to cause pathology.

Although the median HCMV load increased by 1 $\log_{10}$ genomes/ml blood from the time of PCR positivity to retinitis progression in those patients who were PCR positive, there was no constant threshold value above which retinitis would definitely progress. There was also no correlation in each patient between their pre-retinitis HCMV load and their pre-progression load to draw any conclusions as to the degree of viraemia needed in each patient to cause disease. In one patient the HCMV load had peaked prior to retinitis progression and was then undetectable when the progression of retinitis was diagnosed, suggesting that some of the other patients who were PCR negative at progression of their retinitis may have had fluctuations in viral load that were not detected by monthly sampling. More frequent sampling would help to identify such fluctuations in these patients but would obviously impair quality of life. The monthly monitoring of patients on maintenance therapy for HCMV retinitis with HCMV PCR in conjunction with monthly ophthalmological follow-up can help to optimise patients management with regards to the choice of anti-viral therapy and the monitoring for the development of other HCMV related diseases.

The post mortem studies of HCMV load in this chapter confirm the findings in other studies that HCMV is widely distributed throughout many different organs in patients with AIDS but can also be detected in immunocompetent patients (Pillay *et al.* 1993; Webster *et al.* 1995; Toorkey and Carrigan, 1989). However, the results show that the median level of HCMV load is much higher in patients co-infected with HIV compared to HIV negative controls. Moreover, the median HCMV load was greatest in the AIDS patients who had HCMV confirmed histologically and had a background of pre-morbid HCMV disease. Kuhn *et al* have shown similar results from 13 AIDS patients where the patients with histologically proven HCMV encephalitis had viral loads greater than the patients with no

neuropathological evidence of HCMV disease (Kuhn *et al.* 1995). The presence of HCMV in reticuloendothelial tissues, spleen and salivary gland, of the negative controls was not surprising but the detection of HCMV in all three lung samples was. There was however, no prediliction of HCMV for the adrenal gland in the negative controls that is well documented for HIV infected patients. Further studies, including the use of in situ PCR, would help to elucidate the cellular distribution of HIV and HCMV.

CHAPTER 4

A PROSPECTIVE GENOTYPIC ANALYSIS OF UL97 IN AIDS PATIENTS RECEIVING LONG TERM GANCICLOVIR THERAPY

4.1 Introduction

Since the first report of the isolation of HCMV strains resistant to ganciclovir in immunocompromised patients receiving ganciclovir therapy, several studies investigating the development of systemic phenotypic resistance to ganciclovir have been published (Erice *et al.* 1989; Drew *et al.* 1991; Stanat *et al.* 1991; Lurain *et al.* 1992). However, the clinical significance of these phenotypically resistant isolates to the clinical course of the patient with HCMV retinitis has not been established. Moreover, apart from the lengthy process involved in the culturing of HCMV resistant isolates, highly passaged *in vitro* resistant virus and *in vivo* clinical resistance have not been correlated. In 1992, the publication of two elegant molecular studies that identified the UL97 open reading frame as the HCMV gene responsible for the monophosphorylation of ganciclovir made the genotypic study of HCMV resistance a more accessible option (Sullivan *et al.* 1992; Littler *et al.* 1992). This discovery has allowed the development of more rapid molecular techniques, particularly the use of PCR to amplify UL97 from clinical samples and hence to study the evolution of HCMV resistance to ganciclovir without the need for multiple passage in cell culture.

One method that allowed the mapping of AZT resistant mutations in the reverse transcriptase (RT) gene of HIV is the point mutation assay (Kaye *et al.* 1992). This assay was originally developed to detect single nucleotide changes at positions of interest (codons 67, 70, 215 and 219 in HIV-1 RT) in a microtitre format that allows the rapid processing of large numbers of clinical samples to produce "real-time" results. Figure 4.1 illustrates the principles of the assay schematically. The basis of this assay is the amplification of the relevant amplicon with one biotinylated DNA strand (Figure 4.1.1), thus allowing the PCR product to be bound to a streptavidin coated microtitre plate. The captured product is

denatured using NaOH (Figure 4.1.2), and the unbound second strand is washed away. The bound single strand is then annealed with an oligonucleotide probe whose 3' end is one base upstream from the site of the putative point mutation (Figure 4.1.3). Each of the four ^{35}S labelled dNTPs is then added to four separate wells in the presence of a DNA polymerase. If the nucleotide involved in the point mutation is complementary to the added dNTP the annealed probe will be extended by one nucleotide (Figure 4.1.4). Finally the probe and labelled nucleotide are denatured from the target and subjected to liquid scintillation counting (Figure 4.1.5). This sequence is performed in parallel with all four dNTP's to determine the percentage of wildtype or mutant virus present in the population.

This chapter describes the monitoring of patients with HCMV retinitis for the development of mutations in UL97 using PCR based methodology to amplify and sequence the UL97 gene in each patient. The development of a microtitre point mutation assay of UL97 PCR products for the more rapid detection of mutations in UL97 is also described.

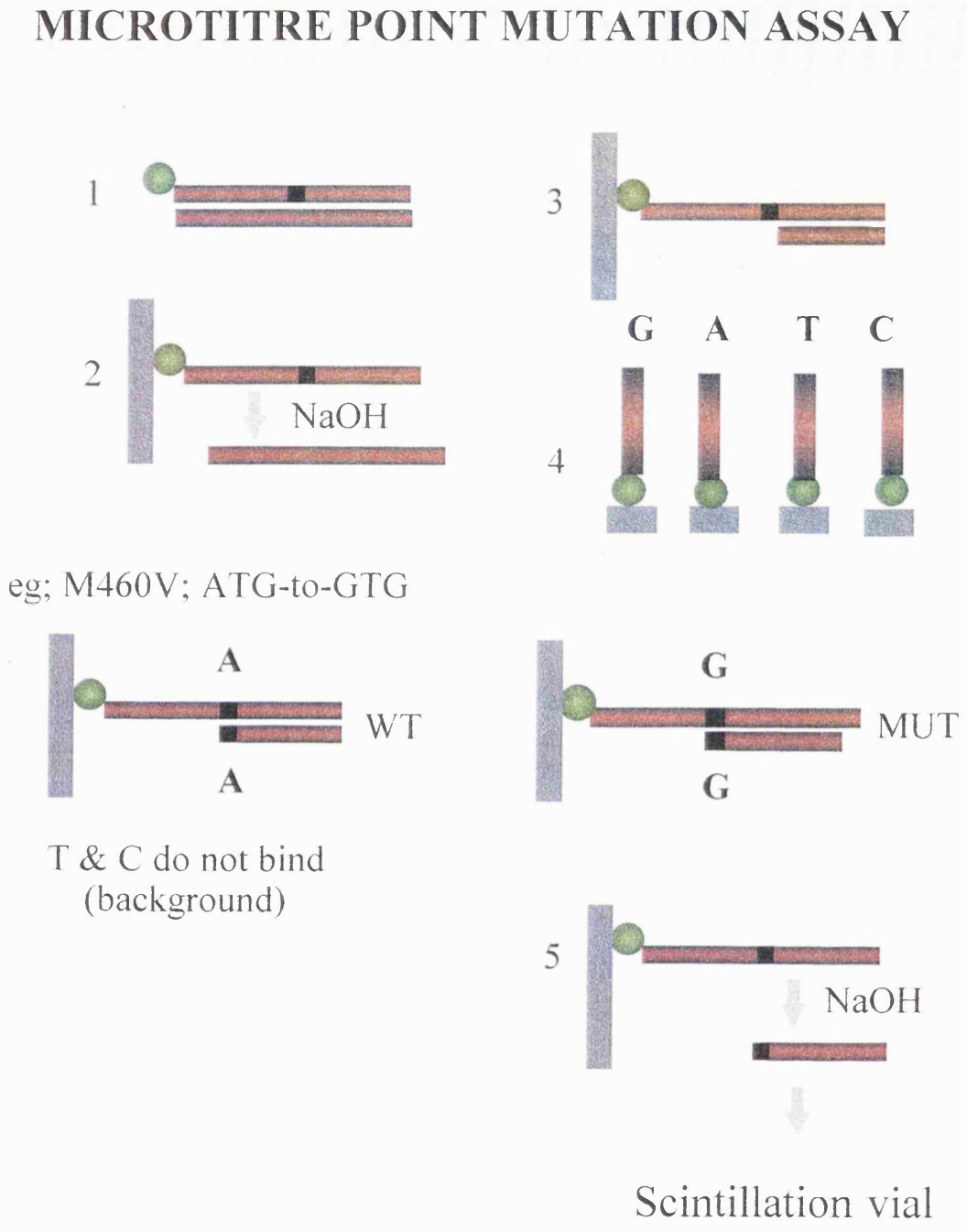

Figure 4.1: The principles of the microtitre point mutation assay. 1) PCR using one biotinylated primer; 2) biotinylated strand binds to streptavidin coated plate; 3) bind single strand with probe; 4) add each of 4 radiolabelled nucleotides to separate wells; 5) denature labelled probe from single strand and count in scintillation counter.

4.2 Methods

4.2.1 Patients

The patient cohort studied for the development of UL97 mutations and the anti-viral therapy they received, is described in section 2.2. All baseline and sequential samples of whole blood, urine and wherever applicable, CSF, vitreous fluid and post mortem tissues were subjected to the qualitative gB PCR assay described in section 2.4. Any sample that gave a positive result for HCMV gB was then subjected to PCR for UL97.

4.3 PCR Amplification of UL97

In order to analyse genotypic changes in the UL97 gene of HCMV a PCR based system for the amplification and sequence analysis of UL97 was devised. The PCR was optimised using DNA extracted from the laboratory strain Ad169 that was propagated in cell culture.

4.3.1 Hirt Extraction of Ad169 HCMV DNA for a Control Sequence for UL97

AD169 was grown in a 25cm^2 tissue culture flask of confluent human embryo lung fibroblasts until 100% CPE was observed (approximately 5-7 days). The cells were washed twice with phosphate buffered saline (PBS), and 0.5ml of lysis buffer (1% sodium dodecyl sulphate (SDS (w/v)), 0.1M Tris-HCl pH 7.5, 10mM EDTA) was added to the monolayer which was then incubated at room temperature for 5 min (Hirt, 1967). The monolayer was then tapped off the bottom of the flask and the lysate transferred to a sterile Eppendorf tube taking care not to shear the DNA. The lysate was then gently mixed with 100µl of 5M NaCl and left at 4^{0}C overnight to precipitate chromosomal DNA. Chromosomal DNA was pelleted by microcentrifugation at 13,000g for 30 min and the supernatant containing

genomic DNA treated with 100µg Proteinase K for 2 hours at 72°C. Viral DNA was subsequently prepared from the supernatant using phenol-chloroform extraction as described in section 3.2.1. DNA was precipitated by the addition of a 2.5x volume of 100% ethanol and 1/10 volume of 4M NaCl at -70°C for at least 2 hours. After centrifugation at 13000g for 20 minutes, the pellet was washed with 70% ethanol and then air dried. Finally, the pellet was resuspended in 20µl of SDW. For optimisation of PCR reactions 1µg of Hirt extracted DNA was used per reaction.

4.3.2 Oligonucleotide Primers Used for the Amplification of UL97

Due to initial difficulties optimising the UL97 PCR on clinical samples a number of different oligonucleotide primer pairs were used both in single round and nested PCR reactions. It was found that a nested PCR was much more consistent in the amplification of UL97 from clinical samples. Figure 4.2 illustrates the position of all the primers used in these different experiments to amplify UL97. All primers were synthesised and HPLC purified commercially by Cruachem (UK). Each primer was supplied in lyophilised form and resuspended in SDW in the clean PCR room to a final concentration of 1µg/µl. The concentration of primer pairs used in the PCR was re-optimised for each new set of primer pairs using concentrations ranging from 50ng to 400ng of each primer.

Figure 4.2: Oligonucleotide primers used to amplify and sequence UL97. Primers used for PCR are red and those used for sequencing are in green (some primers, SC2 & NUL2 were also used for sequencing). The arrows indicate the direction of primer binding.

4.4 Optimisation of the UL97 PCR

Each new set of oligonucleotide primer pairs used the PCR was re-optimised with respect to $MgCl_2$ concentration and the annealing temperature. The optimal $MgCl_2$ concentration was determined as follows; either 1, 1.5, 2, 2.5, 3, 3.5, or 4 mM $MgCl_2$ was added to a reaction mixture of 10x PCR buffer containing 250mM Tris-HCl pH 8.4, 170mM ammonium sulphate, 100mM 2-mercaptoethanol, 0.02% (w/v) gelatin, 200µM of each dNTP and 2.5 units of *Taq* polymerase. The optimal primer concentration was determined by varying the concentration of oligonucleotide primers from 50ng to 400ng in each reaction. The PCR was then set-up as described in section 2.4.2, except that the total volume of the outer PCR (including 5µl target DNA) was 50µl in order to rationalise the use of expensive reagents. Thermal cycling was performed at a range of annealing temperatures to determine the most sensitive annealing temperature for the outer PCR (ranging from 37°C to 55°C) and the most specific temperature for the nested PCR (ranging from 48°C to 60°C). Due to the amplification of a 1060 bp DNA fragment in the outer PCR and a 672 bp product in the nested PCR, each annealing cycle in the thermal cycler was allowed to continue for 2 min and each extension cycle for 3 min. For the optimisation of each new primer pair, DNA extracted from patients was used in dilutions from 10^6 to 10 copies in the outer PCR to assess the sensitivity of the PCR. The copy number for the 672 bp nested product was calculated as follows:

1µg of 1000bp = 1.52 pmoles ⇒ 1µg of 672 = 1.52 ÷ 0.672 = 2.26 pmoles/µg

pmoles/µl = 2.26 × x µg/µl = x pmoles/µl

copies/µl = $x \times 10^{-12} \times 6.022\ 10^{23}$

The amplicons of each reaction were resolved on a 1% agarose gel in the presence of a λ *HindIII/EcoR1* DNA marker as described in section 2.4.3.

4.5 Direct Sequencing of UL97

4.5.1 Purification of UL97 Amplicons Directly After PCR Amplification

UL97 amplicons were purified from a 1% low melting point agarose gel using the Gene Clean II kit (Bio 101, USA) according to the manufacturers instructions. Briefly, the DNA fragment of interest was excised from a low melting point agarose gel and ½ volume of TBE Modifier buffer added followed by 4.5 volumes of sodium iodide stock solution. The gel was melted at 55°C for 5 minutes and 10µl of GLASSMILK suspension was added, mixed and incubated on ice for 5 minutes. The GLASSMILK was pelleted at 13,000*g* for one minute and the supernatant removed. The pellet was washed three times with 700µl of ice cold NEW WASH and the DNA was eluted from the matrix by incubation at 55°C for 3 minutes in 25µl of TE (pH 8.0). A 5µl aliquot of the purified UL97 DNA fragment was then re-analysed on a 1% agarose gel in comparison to a λ *HindIII/EcoR1* DNA marker.

4.5.2 Direct Dideoxy Sequencing of UL97

The purified UL97 DNA was then directly sequenced via the dideoxy chain termination procedure (Sanger *et al.* 1977), using dimethylsulphoxide (DMSO) and the Sequenase 7-deaza-dGTP Sequencing Kit with Sequenase Version 2.0 T7 DNA polymerase (USB). The deaza dGTP kit was used to minimise the formation of compressions due to the high G:C content in the UL97 region of interest. 5µl target DNA (100-200ng) was mixed with 1µl primer (150ng/µl) and 1µl DMSO. The oligonucleotide sequencing primers were internal to the primers used for amplification and are detailed in green in Figure 4.2. The mixture was boiled for 3 min then snap frozen in liquid nitrogen to prevent the denatured double stranded DNA from re-annealing and thus allow primer annealing. To the denatured DNA, 2µl 5x Sequenase reaction buffer (200mM TRIS-HCl pH 7.5, 100mM $MgCl_2$,

250mM NaCl), 2µl nucleotide labelling mix (1.5µM each 7-deaza-dGTP, dTTP, dCTP) diluted 1 in 5 with SDW, 1µl DTT (0.1M solution), 0.5µl [α-S^{35}] dATP (5µCi, Amersham UK), 0.5µl DMSO and 2µl Sequenase enzyme (3 units) diluted 1 in 8 with enzyme dilution buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml BSA) was added. The labelling reaction was allowed to proceed for 5 min at room temperature after which 3.5µl of the reaction mixture was added to each of four Eppendorf tubes containing 2.5µl of each ddNTP and 0.6µl DMSO (pre-warmed to 37°C). Termination was allowed to proceed at 37°C for 5 min before the addition of 1µl nucleotide chase mixture (625µM dNTPs) for a further 5 min at 37°C. At this time 4µl of stop solution (95% (w/v) formamide, 20 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF) was added to each tube and the samples heated to 90°C for 5 min. Completed sequencing reactions were stored at -20°C until they were analysed by polyacrylamide urea gel electrophoresis.

4.5.3 Analysis of DNA Sequence by Polyacrylamide Urea Gel Electrophoresis

A 6% polyacrylamide wedged sequencing gel was prepared as follows; 75g urea (molecular biology grade, Sigma), 22.5ml polyacrylamide (38% acrylamide, 2% bisacrylamide, BDH), 15ml filtered 10x TBE and 52.5ml SDW were dissolved on a heated stirrer for at least 1 hour at 50°C. The container was covered to prevent evaporation of its contents. After dissolution, the mixture was chilled on ice for 20 minutes and 900µl of 10% (w/v) APS and 80µl of TEMED were added. The acrylamide mixture was poured between two 33 x 43 cm (5mm thick) glass plates (BRL) (both cleaned with distilled water and then 70% ethanol) one of which had been coated with dichlorodimethylsilane (Sigma), separated by a 0.4mm plastic spacer (BRL). The gel was wedged at the bottom by the placement of two 20 x 0.4mm plastic spacers to allow enhanced reading of the distal DNA sequences.

Care was taken to prevent air bubbles becoming trapped between the plates. Two 24 well sharks tooth combs were placed in the gel before it had set and 1x TBE was used to remove any unpolymerized acrylamide. The gel was left to polymerise for at least 30 min before being pre-warmed by passing 40W through the gel in 1x TBE for 30 minutes.

5µl of the contents of tubes labelled "G", "A", "T" and "C" were loaded, in that order, into adjacent wells at the top of the gel and were subjected to electrophoresis initially at 40W and then at 60W until the bromophenol blue dye was approximately 3cm from the bottom of the gel. The remaining 5µl of solution in each tube was loaded onto a different set of four adjacent wells at the top of the gel and subjected to electrophoresis until the bromophenol blue of the second loading was 3cm from the bottom of the gel. The gel was then removed from the buffer and the plates carefully separated so that the gel remained attached to the non-siliconised glass plate. This glass plate and the attached gel were soaked in 10% (v/v) glacial acetic acid (BDH) for 30 min. The gel was manually transferred onto Whatman No.3 cartridge paper and vacuum dried at 80°C for approximately 2½ hours on an Atto gel dryer using an Aquavac pump. The dried gel was exposed to X-ray film (Hyperfilm MP: Amersham UK) in a film cassette and placed in a -70°C freezer until processing the film. The exposure time varied from 24 hours to 1 week depending on the activity date of the [α-S^{35}] dATP used for labelling. The DNA sequence was read directly from the film over a light box and compared with the UL97 sequence of Ad169 obtained from the EMBL database (entry code HEHCMVCG).

4.6 Cloning UL97 into pUC18 for Sequencing

4.6.1 Phosphorylation of Primers

In order to sequence UL97, PCR positive samples were re-amplified using phosphorylated nested primers to enable blunt-ended ligation into pUC18 that had been digested with *SmaI* and dephosphorylated. 10µg of each oligonucleotide primer was phosphorylated with T4 Polynucleotide Kinase as described in section 2.5. 1µl of each phosphorylated primer (250ng) was then used in the nested PCR reaction described in section 4.4 and the resulting amplicons analysed on a 1% agarose gel in the presence of a λ *HindIII/EcoR1* DNA marker as described in section 2.4.3.

4.6.2 Ligation of UL97 PCR Products into pUC18

The phosphorylated PCR amplicons were resolved by agarose gel electrophoresis on a 1% low-melting point agarose gel and purified as detailed in section 4.5.1. To enable a blunt ended ligation to take place, any recessed 3' termini of the amplified UL97 products were filled in by the addition of Klenow fragment. 2 units of the Klenow fragment of DNA polymerase 1 were added to the UL97 DNA in the presence of 2µl of 10x MSK buffer (66mM Tris-HCl pH 7.46, 60mM $MgCl_2$, 0.5M NaCl, 10mM DTT) and 4µl 2mM dNTPs in a total volume of 20µl and incubated for 30 minutes at room temperature. The 'filled in' amplicons were subsequently cloned by blunt end ligation into pUC18 that had been *SmaI* digested and dephosphorylated (Boehringer) as follows; approximately 25ng of purified UL97 PCR product was ligated into 75ng of *SmaI* cut dephosphorylated pUC18 vector using 1.25 U of T4 ligase (Northumbria), 1mM ATP (Amersham) in 1µl 10x ligation buffer (0.5M Tris-HCL pH 7.5, 100mM $MgCl_2$, 100mM DDT, 500µg/ml bovine serum albumin), made up to a total of 10µl with SDW. A control ligation was prepared with pUC18 DNA

and all ligation reagents but no insert DNA. The ligations were incubated overnight at 16°C.

4.6.3 Preparation of Transformation Competent *Escherichia coli* (*E. coli*) Cells

10µl of stock JM109 *E. coli* cells, stored at -70°C, were aseptically inoculated into 10ml of sterile Luria Broth (LB, 0.5% (w/v) NaCl (Sigma), 0.5% (w/v) yeast extract (Sigma), 1.0% (w/v) tryptone (Oxoid) and the pH of the broth adjusted to pH 7.5 with NaOH) and incubated at 37°C overnight. 500µl of the overnight culture was aseptically inoculated into 400ml of sterile LB in a 1 litre conical flask and incubated at 37°C on an orbital shaker (150 rpm). 1ml aliquots of the cells were removed at regular intervals and the optical density (OD) of the culture measured in a spectrometer at 600nm until an OD of 0.2 absorbance units was reached (1cm path length) (approximately 4½ hours incubation). The cells were harvested by centrifugation for 5 min at 5,000*g* in sterile 50ml falcon tubes (Nalgene) in an IEC PR7000 centrifuge.

The bacterial cell pellet was resuspended in 200ml of ice cold TFN buffer (50mM $CaCl_2$, 10mM PIPES, 15% glycerol, pH6.6) and incubated on ice for 20 minutes. The cells were harvested by centrifugation, as before, and the bacterial cell pellet was resuspended in 20ml ice cold TFN. 400µl aliquots were dispensed into sterile 1.5ml Eppendorf tubes and snap-frozen in methanol soaked dry ice. The cells were immediately stored at -70°C.

4.6.4 Transformation of Competent *E.coli* with Recombinant pUC18

400µl of competent JM109 cells were thawed on ice. When the cells had just thawed, 100µl of cells were dispensed into pre-cooled 1.5ml Eppendorf tubes containing 10µl of each ligation reaction. As a control for the transformation 1ng of undigested pUC18 was

used in a separate reaction. The cells were incubated on ice for 30 minutes and then subjected to heat-shock at 42°C for 90 seconds. 400µl of LB (prewarmed to 37°C) was added to each Eppendorf tube and the mixture was incubated with shaking at 37°C for 60 minutes. 100µl aliquots of the transformed cells were aseptically streaked onto LB agar plates (3g of bactoagar (Sigma) in 200ml LB, autoclaved then left to cool when 50µg/ml of ampicillin, 40µg/ml 5-bromo 4-chloro 3-indolyl-β-D-galactoside (X-gal) and 46µg/ml isopropyl-β-D-thiogalactopyranoside was added) and incubated overnight at 37°C. Colonies harbouring inserts (white colonies) were selected against the blue colonies containing re-ligated vector only.

4.6.5 Small-scale (Miniprep) Purification of Plasmid DNA

The selected colonies from section 4.6.4 were inoculated into 5ml of LB containing 50µg/ml ampicillin and incubated at 37°C overnight with shaking. The selected colonies were also streaked onto LB/agar plates containing 50µg/ml ampicillin, incubated at 37°C overnight then sealed with Parafilm and stored at +4°C. The overnight bacterial cultures were centrifuged at 3,000*g* in a Beckman TJ6 benchtop centrifuge for 15 minutes, the supernatants were discarded and the bacterial pellets were resuspended in 150µl of Solution I (50mM D-glucose (BDH), 10mM EDTA pH 8.0 (Sigma), 25mM Tris-HCl pH 8.0) in a 1.5ml Eppendorf tube. The cells were then lysed in 300µl of freshly prepared Solution II (0.2M NaOH (Sigma), 1.0% (w/v) SDS (BDH)), and mixed by inversion gently for 5 min. To precipitate cellular DNA, 225µl of Solution III (60ml 5M potassium acetate (BDH), 11.5ml glacial acetic acid (BDH) and 28.5ml SDW) was added, the solution was mixed well and incubated on ice for 5 min. The mixture was microcentrifuged for 10 min at 13,000g to remove all cellular debris. The supernatant was removed and an equal volume of

phenol:chloroform was added, the solution was mixed and microcentrifuged for 5 minutes at 13,000g. The aqueous phase was carefully extracted and 0.6 volumes of isopropanol (BDH) added to precipitate the plasmid DNA. The solution was mixed and centrifuged for 10 min at 13,000*g* in a microcentrifuge. The pellets were washed twice with 300µl 70% ethanol and then dried in air. The plasmid DNA pellets were resuspended in 200µl of TE (pH 8.0) and 5µl of RNAase A (from a 10mg/ml stock of 10mg/ml RNAase A (Boehringer Mannheim), 10mM Tris-HCl (pH 7.5) (Sigma) and 15mM NaCl) was added to each sample followed by incubation at 37°C for 30 min. The plasmid DNA was extracted from the solution with phenol:chloroform (section 4.3.1), precipitated with ethanol and finally vacuum dried. The DNA pellet was resuspended in 20µl of SDW.

When large numbers of clones were being screened simultaneously, ie. >14 clones, the Promega Wizard™ Minipreps DNA Purification system was used (Promega, UK). Selected colonies were grown up overnight in 5ml of LB broth and the cell pellet centrifuged out as described above. The cells were resuspended in 300µl Cell Resuspension Solution (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100µg/ml RNase A), lysed in 300µl Cell Lysis Solution (0.2M NaOH; 1% SDS (w/v)) for 5 min at room temperature and then neutralised by the addition of 300µl Neutralisation Solution (1.32M potassium acetate). The tube was inverted several times and the lysate centrifuged at 13000g in a microcentrifuge for 10 minutes. The resulting supernatant was added with 1ml Wizard™ Minipreps DNA Purification Resin (formula unavailable) into the barrel of a 2ml syringe (Sterilin) attached to a Minicolumn. The syringe plunger was used to push the slurry into the Minicolumn which was subsequently washed in the same way with 2ml Column Wash Solution (80mM potassium acetate; 8.3mM Tris-HCl, pH 7.5; 40µM EDTA and 55% ethanol). The syringe was removed and the Minicolumn placed in a clean 1.5ml Eppendorf tube which was centrifuged

for 2 min to dry the column. The Minicolumn was placed in a clean sterile 1.5ml Eppendorf tube and the DNA eluted in 50µl TE by centrifugation at 13000g for 1 minute.

To confirm the presence of the UL97 insert in pUC18, 5µl of plasmid DNA prepared above was digested with 0.5µl each of *Hind III* and *Eco RI* (both enzymes 10 units/µl) in 1.5µl 10x buffer B (10mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$ 1mM 2-mercaptoethanol) made up to a final volume of 15µl with SDW. The reaction was allowed to proceed at 37°C for 1 hour and the digestion products were analysed on a 1% agarose gel in the presence of a λ *HindIII/EcoRI* DNA marker.

4.7 Plasmid Sequencing of UL97

Plasmid DNA from recombinant colonies was extracted using a standard plasmid mini-preparation procedure (section 4.6.5) and 5µg of DNA subsequently used for sequencing. Five clones from each UL97 PCR were sequenced, and in order to ensure the fidelity of *Taq* polymerase in the amplification of UL97, 5 clones of wildtype UL97 amplified from Ad169 were also subjected to identical PCR amplification conditions and sequencing. The sequence of recombinant clones was determined as described in section 4.5.2 using the Sequenase 7-deaza-dGTP Sequencing Kit and the forward and reverse primers universal to the phage M13 regions of the pUC18 vector along with internal primers detailed in Figure 4.2.

4.7.1 Denaturation of Double-stranded DNA Templates

5µg of the recombinant plasmid DNA in 20µl SDW was denatured by the addition of 5µl 1M NaOH, 1mM EDTA (pH 8.0) and incubated at room temperature for 5 min.

Meanwhile, a CL-6B Sepharose column was prepared by equilibration of CL-6B Sepharose matrix (Sigma) with an equal volume of TE (pH 8.0). The CL-6B Sepharose slurry was used to fill a 0.5ml Eppendorf tube containing a small quantity of glass beads (425-600mm diameter, Sigma). A small hole was pierced in the bottom of the tube with a syringe needle prior to the addition of the beads, and the 0.5ml Eppendorf tube was placed into a 1.5ml Eppendorf tube, also pierced at the bottom with a syringe needle. Both Eppendorf tubes were then placed in a 15ml falcon tube and centrifuged at 400*g* for 5 minutes in a Beckman TJ-6 benchtop centrifuge until the matrix was dry. The large Eppendorf tube was replaced by an intact sterile 1.5 Eppendorf tube. The denatured DNA solution was carefully placed on the top of the matrix and centrifuged at 400*g* for 5 minutes until all of the solution had passed through the column and had been collected in the intact 1.5ml Eppendorf tube.

4.7.2 Annealing of Single Stranded DNA Templates to the Sequencing Primer

The denatured DNA solution from section 4.7.1 (approximately 25µl eluate) was divided into 7µl aliquots and each aliquot added to a separate microcentrifuge tube containing 2µl of 5x Sequenase buffer (USB) and 1µl of primer (either M13 forward/reverse primers or internal primers at a concentration of 50ng/µl). The mixture was incubated at 37°C for 15 minutes, allowed to cool slowly to room temperature and then chilled on ice until used.

The labelling and termination reactions were performed as described in section 4.5.2 except that a shorter labelling period of 2 minutes was used and labelling was performed at 37°C to reduce compressions. The completed sequencing reactions were analysed by polyacrylamide urea gel electrophoresis as in section 4.5.3.

4.8 Point Mutation Assay

4.8.1 PCR Amplification

DNA extracts from whole blood and urine were subjected to nested PCR amplification using the primer sets described in section 4.3. However, for the nested PCR reaction one of the primers in each reaction was biotinylated at the 5' terminus. In order to amplify a UL97 product with a biotinylated sense strand, the biotinylated NUL1 primer was used and, *vice-versa*, biotinylated SC2 was used to amplify a biotinylated anti-sense UL97 product. The nested PCR reaction was carried out using the same $MgCl_2$ concentration and cycling conditions described for the nested UL97 PCR in section 4.4. However, as detailed by Kaye *et al* (Kaye *et al.* 1992), only 50% of the biotinylated primer compared to the unbiotinylated primer is required per PCR reaction. Therefore, 50ng of biotinylated primer was used per reaction compared to 100ng of the other primer.

4.8.2 Design of Olignucleotide Probes

The oligonucleotide probes were designed to bind to the biotinylated strand of the PCR product and finish one base short of the point mutation to be detected. The sequences for the probes were taken from the published sequence of either the sense or anti-sense strand of Ad169. All oligonucleotide probes were synthesised commercially by Cruachem. Probes were made to detect the five most common mutations; M460V, M460I, A594V, L595S and L595F and their sequences are shown in Figure 4.3.

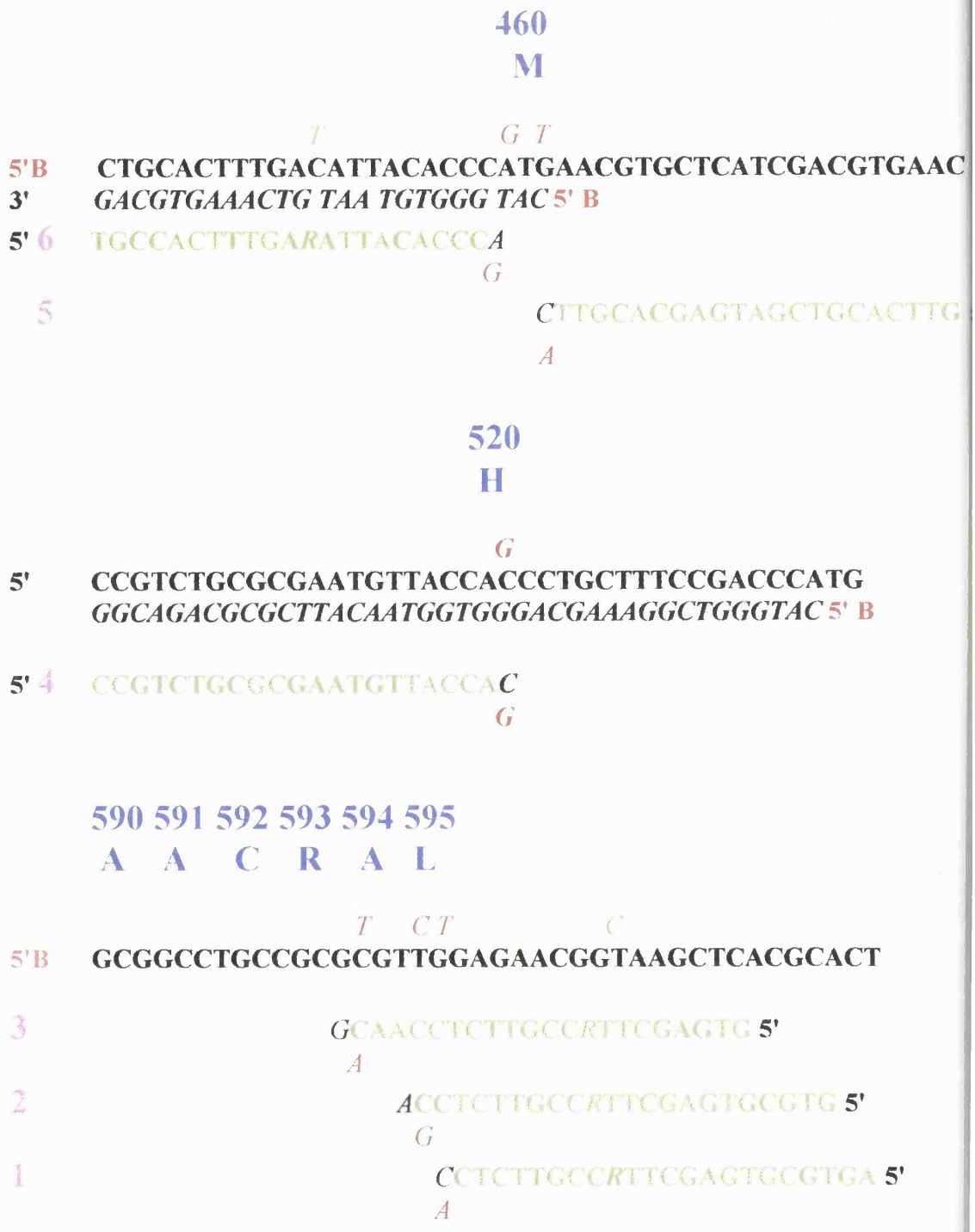

Figure 4.3: Oligonucleotide probes used in the point mutation assay. The probes are in green and numbered 1 to 6. Probes 1 to 3 and 5 bind to the biotinylated sense strand of the PCR product, whilst probes 4 and 6 bind to the biotinylated anti-sense DNA strand. At the 3' end of each probe the black base indicates wildtype and the red base mutant. Silent polymorphisms are shown above the DNA template and *R* base in the probe indicates a mixed base to take into account the silent mutation.

4.8.3 Optimisation of the Microtitre Plate Point Mutation Assay for UL97

In order to optimise the point mutation assay (PMA) for UL97, standard amounts of UL97 DNA of a known sequence were amplified by PCR and subjected to the PMA. For these experiments three separate clones carrying the mutations V594, S595 and F595 were grown and DNA purified. The concentration of DNA in each plasmid preparation was calculated using the OD 260nm of each sample as described in section 4.3.1.

10µl of biotinylated PCR product was diluted to 25µl with 1x TTA buffer (10x TTA; 100mM Tris-HCl pH 7.6, 0.5% Tween 20, 1.0% sodium azide stored at room temperature) and added to each of four microtitre wells of a 96 well streptavidin coated plate (400ng affinity purified streptavidin/300µl well; StrepMax, Advanced Biotechnologies Ltd.) and incubated at 37°C for 60 min. The wells were washed three times with 1x TTA buffer and then 40µl 0.15M NaOH were added to each well and left to incubate for 5 min at room temperature to denature the captured PCR product. The wells were washed four times with 1x TTA to remove the released second strand and 25µl of annealing mix made up of the appropriate oligonucleotide probe in 2.5ml PMA diluent (PMA diluent; 40mM Tris-HCl pH 7.6, 20mM $MgCl_2$, 50mM NaCl stored at 4°C) was added to each well. The oligonucleotide probes (binding to the sense and anti-sense biotinylated strand for all three of the mutations described above) were used in concentrations from 3.3ng to 26ng to maximise probe binding to the biotinylated product whilst keeping non-specific binding to a minimum.

The microtitre plate was covered and placed in a water bath (using different annealing temperatures ranging from 50°C to 65°C to elicit the optimal annealing temperature for these probes) for 3 min and then allowed to cool slowly to room temperature over at least 30 min on a metal block pre-heated to the temperature of the water bath. 24µl of each of the

four ^{35}S-labelled dNTPs (1,200 Ci/mmol stored at -20°C as a 1 in 10 dilution in SDW, NEN Belgium) was added to a 168µl aliquot of 0.1M DTT. To this mixture was added 48µl of DNA polymerase mix (10 units Klenow polymerase, Boehringer Mannheim, diluted in 200µl PMA diluent). A 9.8µl aliquot of each dNTP labelling mix was added to each of the four wells labelled G, A, T, C and incubated at room temperature for 3 min. The wells were washed four times with TTA to remove unincorporated radioactive dNTP from the target strand and 40µl 0.15M NaOH was then added to denature the probe and the incorporated labelled nucleotide. The NaOH solution was removed and mixed with 5ml scintillation cocktail (Optiphase Hisafe-3) in 10ml scintillation vials (Camlab). The radioactivity in the samples was determined in a LKB Rackbeta scintillation counter.

4.8.4 Standardisation of Point Mutation Assay

To generate a standard curve for each of the three probes and their respective mutations known amounts of wildtype (wt) and mutant (mut) sequence were added to each PCR as follows; 100% wt; 75% wt vs 25% mut; 50% wt vs 50% mut; 25% wt vs 75% mut; 100% mut. Each clone could be used as either mut or wt depending on the probe used, therefore acting as its own control. For example, when mixing V594 and S595, probe 3 detected V594 as mut and S595 as wt, whilst probe 2 detected S595 as mut and V594 as wt.

4.9 Results

4.9.1 Optimisation of UL97 PCR

The initial single round UL97 PCR was very efficient at amplifying Hirt extracted Ad169 DNA but very inconsistent at amplifying UL97 from clinical samples. Therefore, all subsequent new primer sets and conditions were optimised in a nested PCR using DNA extracted from clinical samples (whole blood).

Following the optimisation described in section 4.4, the primer pairs UL1 & UL2 for the outer PCR and NUL1 & SC2 for the nested PCR, were able to amplify the UL97 sequence from the majority of clinical samples. The optimal magnesium chloride concentration was determined for the above primer sets and was found to be 3mM for the outer PCR and 1mM for the nested PCR as shown in Figure 4.4. The optimal oligonucleotide primer concentration was determined for both the outer and nested PCR reactions and found to be 100ng as shown in Figure 4.4. The optimal annealing temperature for the outer PCR was 45°C and for the nested PCR 58°C. Figure 4.5 shows the increased sensitivity of the outer PCR when using an annealing temperature of 45°C compared to 50°C when both PCRs were subjected to nesting at an annealing temperature of 58°C. At an annealing temperature of 45°C in the outer PCR, 10 copies of UL97 could be amplified from clinical samples. Despite the above optimisation of this PCR there still remained some clinical samples, mostly from two patients on ganciclovir maintenance therapy, where UL97 could not be amplified. This accounted for approximately 5% of clinical samples available for UL97 analysis.

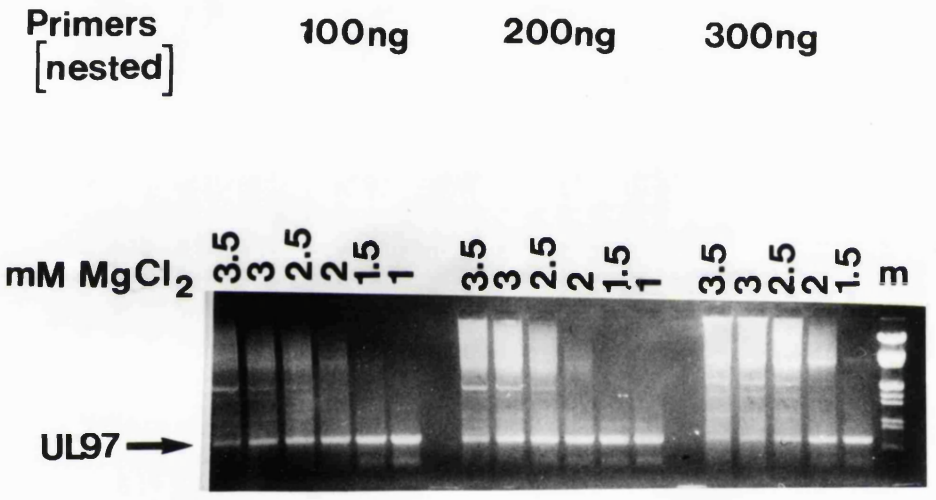

Figure 4.4: Optimisation of magnesium and primer concentrations for UL97 PCR in the presence of a λ *HindIII/EcoRI* DNA marker (m).

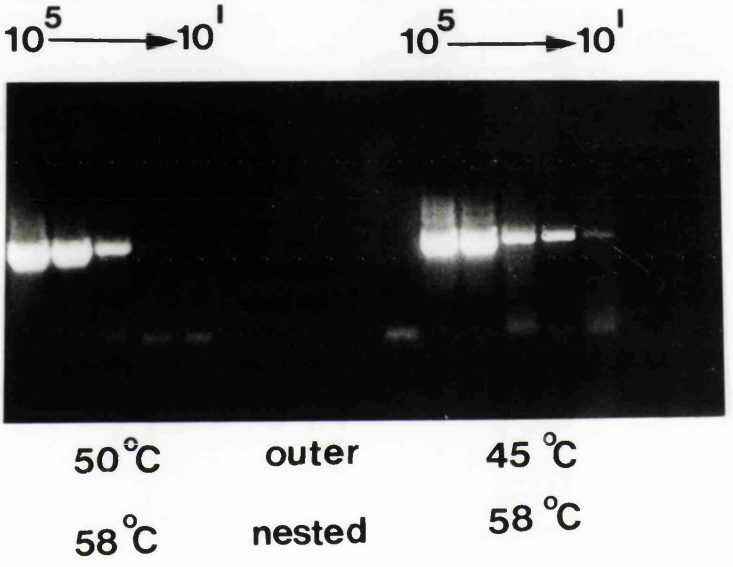

Figure 4.5: Optimisation of the outer PCR annealing temperature (at a constant nested annealing temperature) for UL97 PCR using patient DNA at different copy numbers in the presence of a λ *HindIII/EcoRI* DNA marker (m).

4.10 Direct Sequencing of UL97 PCR Products

The purified UL97 amplicons were sequenced using internal primers spaced approximately 200 bp apart. Therefore, allowing for the reading of up to 250 bp from each primer, the entire UL97 amplicon was sequenced in overlapping regions. Due to the high G:C content of the UL97 region there were several areas of compression that were impossible to read using conventional DNA sequencing. This problem was overcome by using the Sequenase 7-deaza-dGTP Sequencing Kit with Sequenase Version 2.0 T7 DNA polymerase. Compressions occur when DNA sequences rich in dG and dC residues are not fully denatured during electrophoresis thus interfering with the normal migration patterns of DNA fragments in a denaturing gel. This may manifest as either the compression of several bands together or bands further apart than normal and can be reduced by the substitution of dGTP with either dITP or 7-deaza-dGTP which form weaker secondary structures. Despite multiple attempts, no success was achieved when using dITP. However, the use of the 7-deaza-dGTP kit was successful in greatly reducing the degree of compressions as shown in Figure 4.6. Whilst large regions of UL97 DNA could be read using direct sequencing, the areas of most interest were difficult to interpret due to mixed populations of DNA and residual compressions. I therefore decided to determine the sequence of the UL97 amplicons following cloning into pUC18.

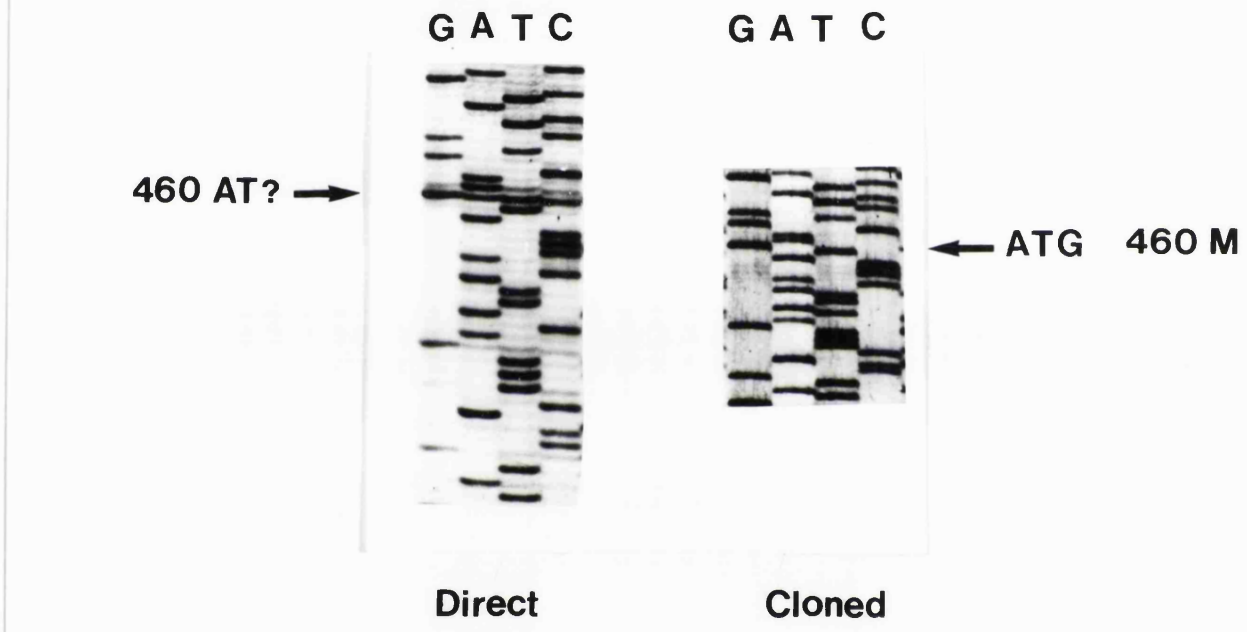

Figure 4.6: Cloned vs direct sequencing to read a difficult area of compression around the 460 codon of UL97.

4.11 Plasmid Sequencing of the UL97 Region

4.11.1 Plasmid Sequencing of UL97 from Ad169

Sequencing of five clones of UL97 from Hirt extracted Ad169 revealed five sequences identical to that described by Chee *et al* (Chee MS. and Bankier AT. 1990). There was no evidence of mutations introduced into the sequence by *Taq* infidelity in the two round PCR amplification used for UL97. There were several silent point mutations that were found to recur in many pre and post-treatment clinical samples and always in all five clones of each sample. Most of these polymorphisms have been previously reported in both clinical samples and in the HCMV laboratory strains Toledo and Towne.

4.11.2 Plasmid Sequencing of Ad169 in Clinical Samples

Twenty one patients were HCMV PCR positive in blood at some stage during follow-up. Seven patients had their pre and post treatment UL97 sequences (and in two of these patients multiple tissues obtained at post-mortem) fully analysed by cloned sequence analysis of their UL97 region cloned into pUC18. In addition to the seven patients with HCMV retinitis from this cohort, one extra patient, a young woman with HCMV polyradiculopathy, had pre and post-mortem samples sequenced. The UL97 sequences of the remaining 14 patients were determined using the point mutation assay and the results are described in section 4.12.3.

Three patients were found to have mutations that have previously been shown to confer ganciclovir resistance to Ad169. These mutations were; F595, S595 and V594 as shown in Figures 4.7 and 4.8 respectively. The two 595 mutations (in patients LM and RJ, see Tables 4.1.1 and 4.1.2) were associated with the first episode of retinitis progression in two

patients on ganciclovir (at 241 and 127 days of treatment respectively). Both patients had wildtype 595 pre ganciclovir therapy. A more detailed study of the evolution of these two mutations and their impact on viral fitness is described in Chapter 5. The V594 mutation was found in DNA extracted from the lung of a patient (DW) who had been on ganciclovir for 4 months and had died of bronchopneumonia. This mutation was not found in any other blood or tissue samples (Table 4.1.3).

Several other coding mutations that have not been described previously were found. Two patients (DW and MT) had coding mutations present in their pre-treatment blood samples. Neither patient became HCMV PCR negative after ganciclovir induction therapy. One patient, MT, had a coding mutation at H469L that was also found at his first episode of retinitis progression but was not found in an interim sample after induction therapy (see Table 4.1.4 and Figure 3.3). The second patient, DW, had a coding mutation at codon Q449L at the time of retinitis diagnosis and first progression of retinitis. This Q449L mutation was not found in any tissue samples at post mortem. DW was also found to have coding mutations at PM in the oesophagus and lung. The third patient, AV, was also found to have a coding mutation at R686W in UL97 before ganciclovir therapy for HCMV polyradiculitis (Table 4.1.5). She became PCR negative in blood following ganciclovir therapy but her radiculopathy progressed and she died of acute encephalitis. At PM she was found to have a very high HCMV load in spinal cord (6.79 $\log_{10}$ genomes/μg DNA) and several coding mutations were also present (Table 4.1.5). Of the remaining three patients, one, DM, had a coding mutation at 465 at the time of retinitis progression and died shortly after re-induction with ganciclovir. Patients MA and JH had wildtype UL97 sequences throughout their treatment course and, for JH, at PM. Both patients received cidofovir (see Figures 3.4 & 3.5) and their UL54 genes are currently undergoing sequence analysis.

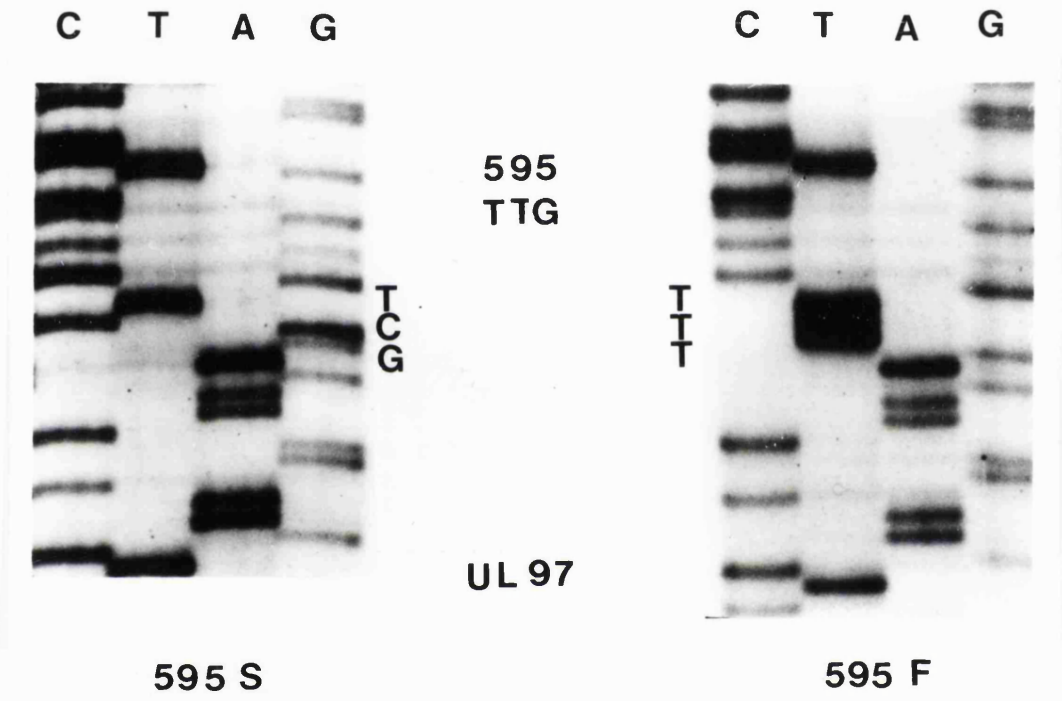

Figure 4.7: Autoradiograph of the UL97 sequence from two patients on ganciclovir showing mutations at S595 and F595. Both samples were taken at the time of first progression of retinitis.

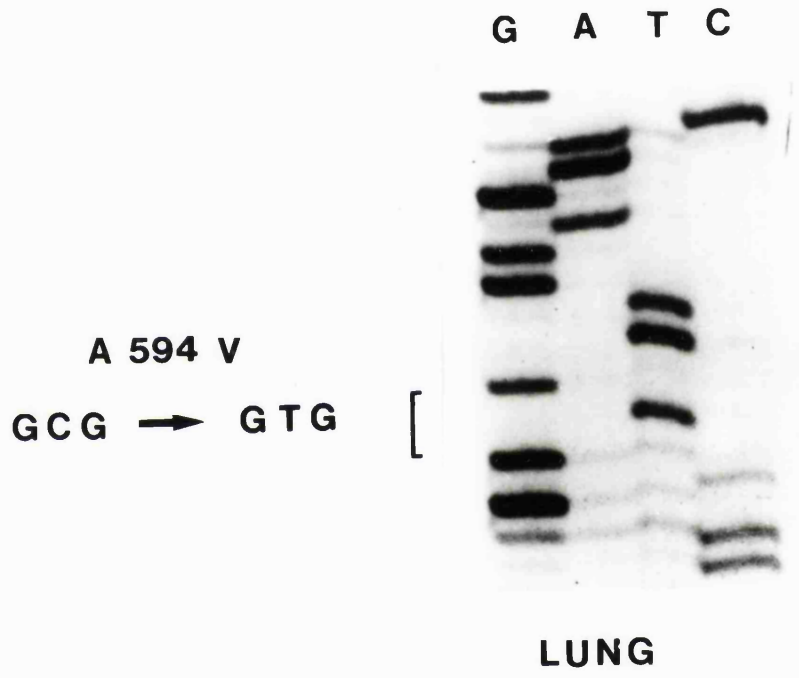

Figure 4.8: Autoradiograph of the UL97 sequence from the lung of a patient with HCMV retinitis who received ganciclovir for 5 months before dying from bronchopneumonia.

Tables 4.1.1-4.1.8: The distribution of mutations found by the sequencing of UL97 clones in eight patients who received ganciclovir therapy. The nucleotide change is indicated as well as any coding mutation and the change in amino acid. Clinical events (RP=retinitis progression, AI=adrenal insufficiency), drug therapy and location of sample are all indicated.

Table 4.1.1 LM		**CODON**						
		503	509	525	544	579	595	598
SAMPLE	**DRUG**	1509	1527	1575	1632	1737	1785	1794
Blood 27/7/95 Retinitis	-	T→C	C→T	C→T		C→T		T→C
Blood 27/10/95	GCV	T→C	C→T		C→T	C→T	G→T	T→C
Blood 30/11/95 1st RP	GCV	T→C	C→T		C→T	C→T	G→T	T→C
							Leu ↓ Phe	

Table 4.1.2 RJ		**CODON**					
		456	503	579	595	598	616
SAMPLE	**DRUG**	1368	1509	1737	1784	1794	1848
Blood 20/12/94 Retinitis	-	C→T	T→C	C→T		T→C	
Blood 17/6/95	GCV			C→T	T→C	T→C	C→T
Blood 18/8/95 1st RP	GCV	C→T		C→T	T→C	T→C	C→T
					Leu ↓ Ser		

Table 4.1.3 DW	CODON							
	449	456	553	594	598	599	611	702
SAMPLE	1346	1366	1657	1781	1794	1811	1797	2106
Urine 14/4/93			C→T		T→C			C→T
Blood 29/11/93: Retinitis	C→T				T→C		C→T	C→T
Blood 31/12/93 1st RP	C→T							
Oesophagus		G→A	C→T		T→C			C→T
Adrenal			C→T		T→C			C→T
Lymph node			C→T		T→C			C→T
Lung				C→T		A→G		C→T
	Gln ↓ Leu	Arg ↓ Asn		Ala ↓ Val		Lys ↓ Asn		

Table 4.1.4 MT		CODON					
		456	469	579	598	683	702
SAMPLE	DRUG	1368	1406	1737	1794	2049	2106
Blood 2/9/93 Retinitis	-	C→T	C→T	C→T	T→C	C→T	
Blood 24/9/93 Post IT	GCV	C→T		C→T	T→C	C→T	C→T
Blood 18/11/93 1st RP	GCV	C→T	C→T	C→T	T→C	C→T	C→T
			His ↓ Leu				

Table 4.1.5 AV	CODON												
	456	487	489	503	525	553	571	579	598	604	611	686	702
TISSUE	1368	1461	1466	1509	1575	1657	1711	1737	1794	1811	1848	2056	2106
Blood 11/3/94 Polyradiculopathy	C→T			T→C	C→T	C→T		C→T	T→C			C→T	C→T
DIED 31/5/94: Spinal Cord		C→T	C→T					C→T	T→C	C→T	C→T	C→T	C→T
Brain	C→T					C→T		C→T	T→C				C→T
Adrenal				T→C	C→T	C→T	C→T	C→T	T→C				C→T
Lymph node						C→T		C→T	T→C				
			Pro ↓ Leu				Arg ↓ Cys			Ser ↓ Phe		Arg ↓ Trp	

Table 4.1.6 DM		CODON						
		456	465	553	579	598	683	702
SAMPLE	DRUG	1368	1393	1657	1737	1794	2049	2106
Blood 2/9/93 Retinitis	-	C→T		C→T	C→T	T→C	C→T	C→T
Blood 18/11/93 1st RP	GCV	C→T	G→A	C→T	C→T	T→C	C→T	C→T
			Asp ↓ Asn					

Table 4.1.7 MA		CODON		
		456	598	616
SAMPLE	DRUG	1368	1794	1848
Urine 15/11/94 Retinitis	-	C→T	T→C	C→T
Blood 17/3/95 2nd RP	GCV non-compliant	C→T	T→C	C→T
Blood 7/6/95 AI	CDV	C→T		C→T

Table 4.1.8 JH		CODON						
		456	487	553	579	584	598	611
SAMPLE	DRUG	1368	1461	1657	1737	1752	1794	1831
Blood 29/11/94 Retinitis	-	C→T	C→G	C→T	C→T		T→C	
Blood 7/6/95	GCV 1/12 CDV 5/12		C→G		C→T	C→T	T→C	C→T
Died 15/6/95								
Brain		C→T		C→T	C→T		T→C	
L vitreous		C→T			C→T	C→T		
R vitreous		C→T		C→T	C→T		T→C	
Adrenal				C→T	C→T		T→C	C→T
Lymph node				C→T	C→T		T→C	
Lung		C→T			C→T		T→C	C→T

4.12 Point Mutation Assay for UL97 Drug Resistant Mutations

4.12.1 Optimisation of the PMA

The optimal concentration of oligonucleotide probe for UL97 was 6.6ng, as for the HIV PMA. Although greater probe concentrations gave stronger signals, the non-specific background amplification was also very much higher. Therefore, each probe was stored at a concentration of 66ng and diluted 1 in 10 with SDW before mixing with 2.5ml PMA diluent.

An annealing temperature of 63°C, as used for the HIV PMA, was too high for adequate binding of the probes to UL97. Different annealing temperatures were tested (45°C, 50°C, 55°C and 60°C) and it was found that maximal binding of probe, with minimal background signal, was achieved at an annealing temperature of 55°C followed by slowly cooling the plate down to room temperature on a metal block that had also been heated to 55°C.

The three oligonucleotide probes for the 595 and 594 mutations only bound when using the biotinylated sense strand of the UL97 amplicons and the anti-sense probes, ie the biotinylated antisense UL97 DNA and the sense probes did not give a signal for any dNTPs. This was probably due to inherent secondary structure in the bases encoding aa 590-595 which was also found to be a problem during sequencing of that region. All three antisense probes contained one mixed nucleotide base at position 1794 where the Ad169 sequence is a T but the majority of clones from clinical samples exhibited a silent mutation to C. This mixed base did not affect the binding as the results obtained from all three probes were consistent.

4.12.2 Standardisation of PMA

Three plasmids each containing a single UL97 mutation were used to produce a standard curve by mixing known concentrations of each plasmid prior to the PCR. As each plasmid already contained the UL97 insert only the nested PCR was required to biotinylate one of the DNA strands. Due to the large amount of DNA present in each plasmid, 5ng of each plasmid was used for each reaction. Depending on the probe used each plasmid could act both as "mutant" or "wildtype" sequences. The standard curves for the three mutations F595, S595 and V594, with their respective oligonucleotide probes 1,2 and 3, are shown in Figure 4.9.

The correlation coefficients for the three probes were;

Probe 1 L595F R = 0.9997

Probe 2 L595S R = 0.9978

Probe 3 A594V R = 0.9969

As I had not detected either the Q520, I460 or V460 mutations in any of the other five patients sequenced, I was unable to establish a standard curve for the detection of these mutations using their respective probes 4, 5 and 6. I did, however, use these probes on patient samples that had already been sequenced by plasmid sequencing and were known to be wildtype at H520 and M460. All three probes identified these UL97 sequences from clinical samples as wildtype.

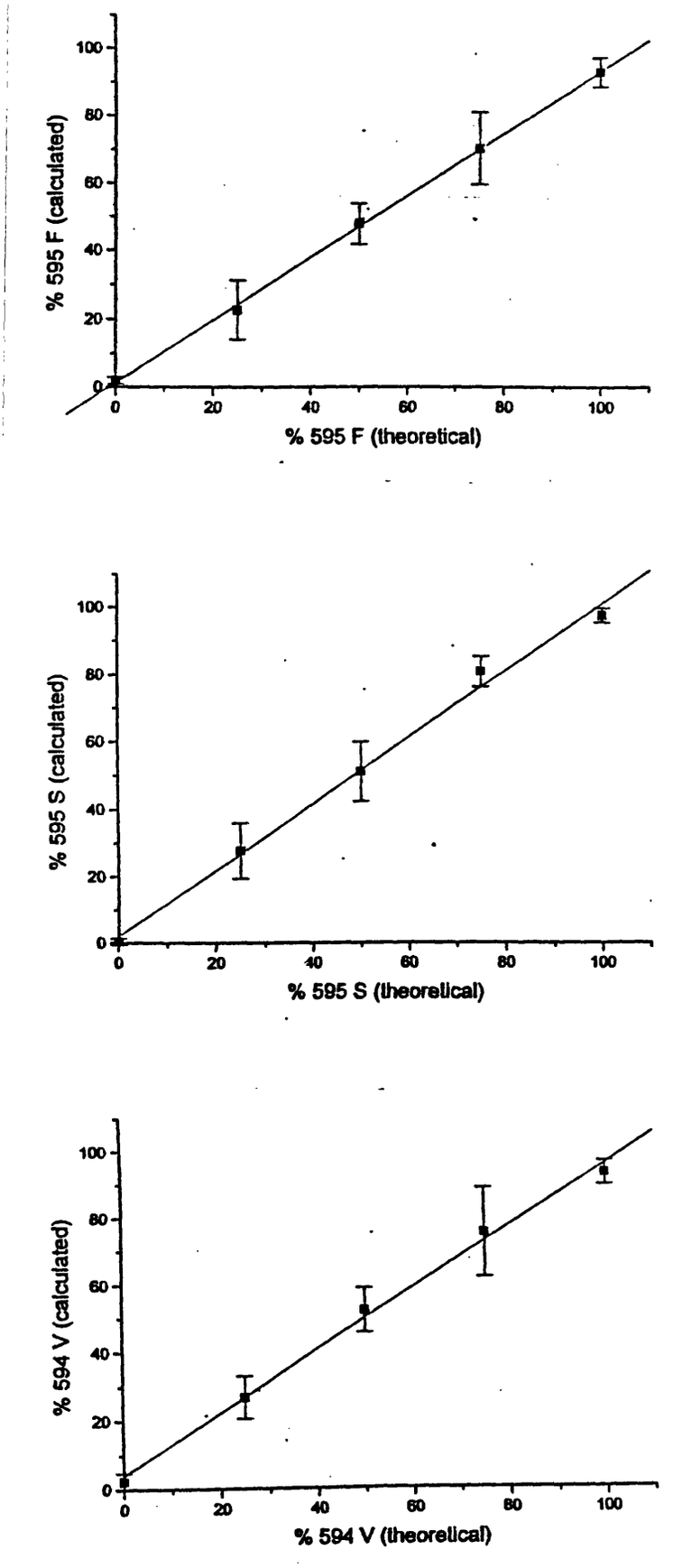

Figure 4.9: The standard curves for probes 1, 2, and 3 for detecting F595, S595 and V594 respectively.

4.12.3 UL97 Sequence using the Point Mutation Assay

Fourteen patients had sequential samples analysed by the point mutation assay for the five most common coding mutations that have been shown to transfer ganciclovir resistance to Ad169. Where available, pre-treatment samples were also analysed by the PMA. Table 4.2 shows the percentage mutant virus present at each nucleotide base for each patients sample (where a sample was unavailable N/A is indicated). The current anti-HCMV therapy at time of sample is indicated along with any clinical events. If the first event noted is the second episode of retinitis progression then the patient was PCR negative at first progression.

There is an even distribution of mutations at the three codons 460, 594 and 595 with only one patient found exhibiting a 520 mutation. The three patients (TE, AH and BP) with >2 concurrent mutations experienced very progressive retinitis affecting both eyes. Three of the four patients (PB, JLF, DS) who were PCR positive at first progression of retinitis were found to have mutations at the time of progression. However, this was not the case for every patient (PR) and some remained wildtype despite several progressions (MG, GN, RM, FP and MY). The two patients who developed HCMV disease in the central/peripheral nervous system had wildtype UL97 both in the blood (LR) and CSF (LR, FP).

Table 4.2: Distribution of 5 common mutations found in UL97 by a point mutation assay

Pt.	HCMV Dx (days)	DRUG	L595F %F	L595S %S	A594V %V	H520Q %Q	M460I %I	M460V %V
PB	Retinitis	-	N/A	N/A	N/A	N/A	N/A	N/A
	1st RP (128)	GCV	wt,	wt	wt	wt	wt	54%
PR	Retinitis	-	wt	wt	wt	wt	wt	wt
	1st RP (137)	GCV	wt	10%	4%	wt	5%	wt
MG	Retinitis	-	N/A	N/A	N/A	N/A	N/A	N/A
	3rd RP (188)	PFA	wt	wt	19%	wt	wt	wt
TE	Retinitis	-	wt	wt	wt	wt	wt	wt
	1st RP (119)	GCV	8%	wt	wt	wt	24%	wt
	2nd RP (148)	GCV	26%	wt	wt	wt	31%	wt
	3rd RP (218)	PFA	15%	wt	wt	wt	25%	wt
	GID (458)	PFA	wt	wt	wt	wt	29%	wt
	GID/AI (486)	GCV	wt	43%	wt	wt	18%	wt
JLF	Retinitis	-	N/A	N/A	N/A	N/A	N/A	N/A
	1st RP (120)	GCV	wt	wt	wt	92%	wt	wt
AH	Retinitis	-	wt	wt	wt	wt	wt	wt
	1st RP (294)	GCV	DNB*	DNB	DNB	wt	wt	wt
	2nd RP (359)	PFA	DNB	DNB	DNB	wt	wt	wt
		CDV	10%	wt	18%	wt	wt	12%
	AI (450)	CDV	45%	wt	15%	wt	wt	5%
RM	Retinitis	-	wt	wt	wt	wt	wt	wt
	1st RP (151)	GCV	wt	wt	wt	wt	wt	wt
LR	Retinitis	-	wt	wt	wt	wt	wt	wt
	CNS; (361)	GCV	wt	14%	12%	wt	4%	wt
	CSF		wt	7%	11%	wt	5%	wt
FP	Retinitis	-	wt	wt	wt	wt	wt	wt
	PNS:CSF(143)	GCV	wt	wt	wt	wt	9%	wt
DS	Retinitis	-	N/A	N/A	N/A	N/A	N/A	N/A
	1st RP (113)	GCV	wt	49%	wt	wt	wt	wt
	CNS; (146)	GCV	wt	95%	wt	wt	wt	wt
GN	Retinitis	-	(PCR-)	-	-	-	-	-
	3rd RP (605)	GCV	20%	wt	wt	wt	wt	wt
	L vitreous (605)	GCV	wt	wt	wt	wt	wt	wt
AG	Retinitis	-	wt	wt	wt	wt	wt	wt
	1st RP (76)	GCV	wt	wt	wt	wt	wt	wt
MY	Retinitis	-	-	-	-	-	-	-
	AI (364)	PFA	wt	wt	wt	wt	wt	wt
BP	Retinitis	-	wt	wt	wt	wt	wt	wt
	(88)	CDV	wt	wt	wt	wt	wt	wt
	3rd RP (394)	GCV	31%	wt	95%	wt	50%	wt
	(426)	GCV	26%	wt	98%	wt	70%	wt

In one patient (AH; see Table 4.2 for PMA data and Figure 3.4 for viral load profile), the oligonucleotide probes 1-3 did not bind to the UL97 sequences corresponding to amino acids 595 and 595 although the UL97 sequence was wildtype at codons 520 and 460. As part of a collaborative project with Gilead Sciences (San Francisco) studying the relationship between HCMV viral load and UL97 mutations in patients receiving cidofovir, aliquots of this patient's samples had been sent to their laboratory. They found that in the first two samples associated with retinitis progression, whilst the patient was on ganciclovir, there was a 44 bp deletion between nucleotides 1767 to 1811 as shown in Figure 4.10 (photograph courtesy of Dr J Cherrington, Gilead Sciences, California). This UL97 deletion mutant had reverted to wildtype in samples 3 and 4 concomitant with stabilisation of the retinitis by cidofovir.

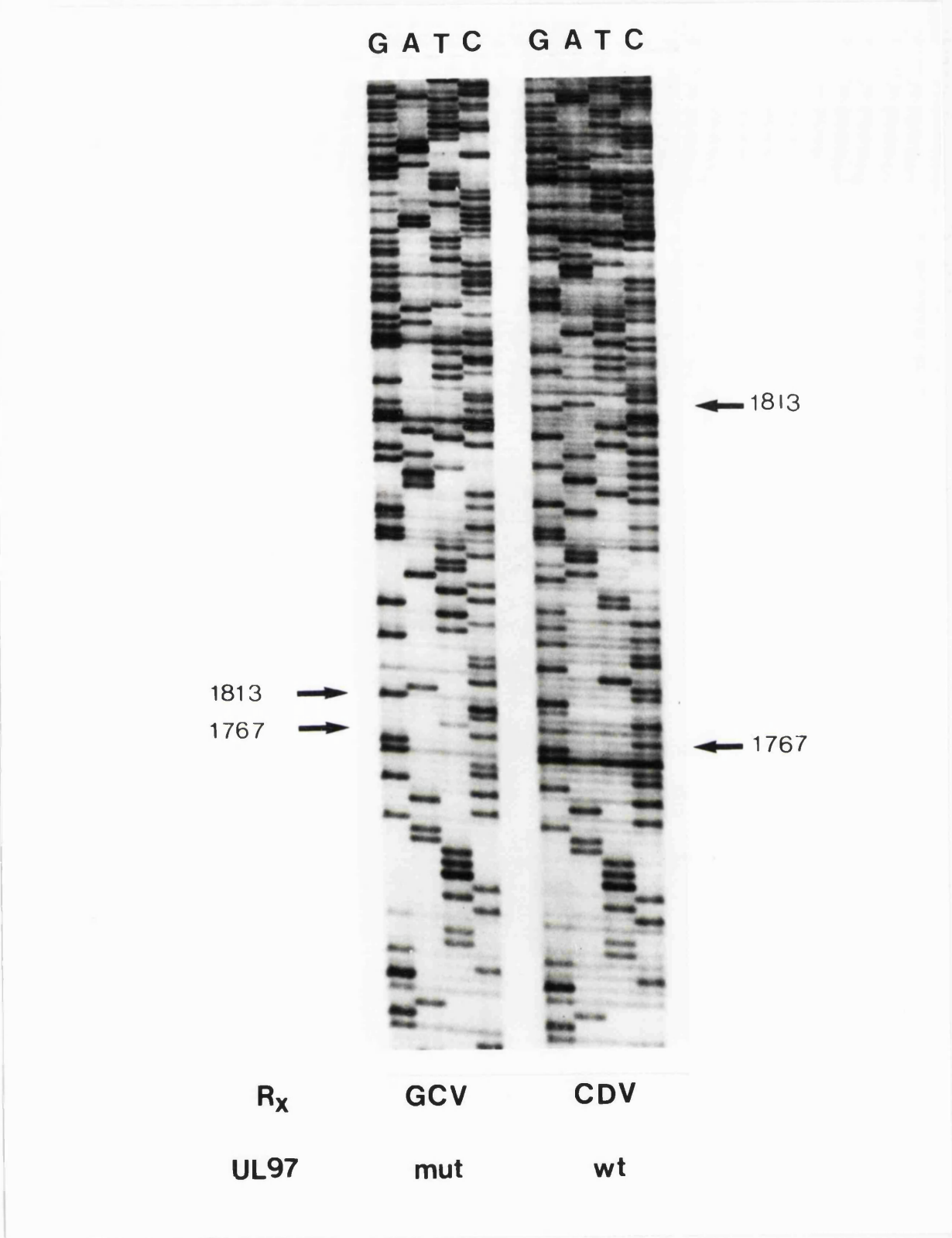

Figure 4.10: Autoradiograph of 44 bp deletion (between bp 1767 to 1813) in UL97 at first retinitis progression whilst on ganciclovir(GCV) therapy. UL97 reverted to wildtype after switching to cidofovir (CDV) therapy. Photograph courtesy of Dr J Cherrington, Gilead Sciences, California.

4.13 Discussion

Since the UL97 ORF was identified in 1992 as the gene responsible for the monophosphorylation of ganciclovir, several different molecular methods have been developed to amplify and sequence UL97 directly from clinical samples of patients receiving ganciclovir (Alain *et al.* 1995; Wolf and Spector, 1992). I have developed a nested PCR reaction that can amplify UL97 from the majority of gB PCR positive samples, including blood, urine, CSF, vitreous and post mortem tissues. The PCR was, however, difficult to optimise and gave very different sensitivities for DNA extracted from Ad169 compared to DNA from clinical samples. The reasons for this difference in success of amplification from laboratory and clinically derived material remains unclear. Undoubtedly, the absolute copy number of HCMV DNA is higher in the Hirt extracted DNA but the quantity of HCMV DNA in some clinical samples was known to be above 5 $\log_{10}$ genomes/ml from gB QCPCR and the UL97 gene still could not be amplified. The reasons for this observation could be two-fold, either mutations in the primer binding sites within UL97 reduced the efficiency of the amplification, or inhibitory substances were present in the extracted DNA. The presence of UL97 mutations may have been a cause in the samples from patients on long term ganciclovir therapy but not on pre-treatment ganciclovir naive patients. Every sample was processed neat and at 1 in 20 dilutions and, in some instances to counteract the possible presence of inhibitors, dilutions of up to 1 in 1600 were used to no avail. UL97 is a difficult region to sequence due to the high degree of secondary structure and this may have had the major impact on primer binding and efficient amplification in the PCR.

In the seven patients whose UL97 sequences were determined by double stranded DNA sequencing several new coding mutations were identified. The presence of these mutations in two pre-treatment samples correlated with a reduced response to ganciclovir induction therapy. Neither mutation has been shown to transfer ganciclovir resistance to UL97 but both mutations, H469L and Q449L, were in close proximity to subdomain VI that is thought to be involved in substrate recognition and where mutations at codon 460 have been shown by marker transfer experiments to confer resistance to Ad169. The presence of widespread different coding mutations in the post mortem samples suggests that there may be varying selection pressures in different organs. Further studies, including biochemical studies on the nature of these mutations, with a larger number of patients would be needed to investigate this theory.

The cloning of PCR products and their subsequent sequencing is a time consuming process and more rapid techniques for the determination of the UL97 sequence have been published. Chou *et al* first analysed, by conventional sequencing methods, isolates from 10 patients known to be phenotypically resistant to ganciclovir, and found that all 10 mutations involved one of three codons; 4 were mutant at V460, 3 at S595, 2 at V594 and 1 at F595 (Chou *et al.* 1995a). Further analysis of a different 30 clinical isolates showed that restriction enzyme analysis (using the restriction enzymes *Nla*III to detect mutations at M460, *Hha*I to detect changes at A594 and *Taq*I to detect the L595S mutation) of the codons 460, 594 and S595 (F595 could not be detected by this method) could identify the mutations present in >73% of the isolates with a ganciclovir IC_{50} >6.0μM (Chou *et al.* 1995b). Using these results I decided to base the point mutation assay on these three codons and also included codon 520 (which was responsible for one of the mutant isolates in the above study). However, unlike

both studies by Chou *et al*, all of the samples analysed by the point mutation assay had not been passaged in cell culture. My results are very similar to those reported by Chou *et al* since an even distribution of mutations throughout the three major codons associated with ganciclovir resistance was found (Chou *et al.* 1995b).

Whilst describing the prevalence of ganciclovir resistant mutations in known resistant isolates, Chou *et al* did not attempt to correlate these mutations with clinical events. Bovin *et al* described four patients (2 with AIDS and 2 with chronic lymphocytic leukaemia) with progressive disseminated HCMV disease who were found have mutations at codons 460, 594 and 595(S) directly amplified from DNA extracted from PMNLs (Boivin *et al.* 1996). These mutations were found to correlate with the mutations found in isolates recovered at the same time. The results in this thesis suggest that in the majority of cases the finding of mutant virus correlates with clinical events in patients who are PCR positive. In the 14 patients who were PCR positive in blood at first retinitis progression, 10 patients were found to have mutations in UL97 (5 by PMA and 5 by cloned sequencing) and none of these 10 patients became PCR negative following ganciclovir re-induction therapy. Of the other 4 patients who were PCR positive at progression all 4 were found to have wildtype UL97 at that time (by PMA), three patients became PCR negative with intravenous ganciclovir whilst the fourth died shortly after his retinitis progression. 25 patients, however, remained PCR negative and still had progressive retinitis suggesting that reduced susceptibility of HCMV in the blood is not always the reason for HCMV disease progression. The point mutation assay was also effective at amplifying UL97 from CSF and vitreous fluid. Further studies analysing UL97 sequence in the vitreous humour would be much more relevant and informative as to the role that resistant virus may play in localised disease progression.

Obtaining vitreous samples for study may soon become more feasible, with the increased use of intraocular injections and intraocular ganciclovir implants.

The point mutation assay is a rapid (24 hour turnaround) and reproducible means of studying the genetic changes in UL97 amplified directly from clinical samples. It is a real time assay that can be used to not only to optimise patients maintenance therapy but also to enhance our understanding of the viral fitness of different UL97 mutations as discussed in the following chapter.

CHAPTER 5

DYNAMICS OF HCMV INFECTION IN THE PRESENCE OF ANTI-VIRAL THERAPY

5.1 Introduction

Despite the progress made in the understanding of the dynamics of HIV infection in the human host through the application of mathematical modelling (Ho *et al.* 1995) (Wei *et al.* 1995), there is a paucity of data on the replication kinetics of HCMV infection *in vivo*. Moreover, with the increasing emergence of HCMV strains carrying mutations either in UL97 or DNA polymerase that confer resistance to ganciclovir and other anti-viral agents, there is very little data on the relative fitness of such strains. Extension of the arguments developed by Coffin for HIV, predicts that drug resistant mutants of HCMV are likely to replicate more slowly otherwise the mutations would be frequently detected in untreated individuals (Coffin, 1995). In this chapter these theories have been extended to the study of HCMV strains carrying UL97 mutations.

This chapter describes the application of results obtained in this thesis to the understanding of the *in vivo* dynamics of HCMV infection; firstly in response to ganciclovir induction therapy and then in relation to the presence of drug resistant mutations and their implications for viral fitness.

5.2 Methods

The patient population studied and their HCMV load results (determined in blood and urine by QCPCR as described in section 2.5) have been discussed in sections 2.2 and 2.9 respectively. The half-life for HCMV was calculated for all 39 patients who were HCMV PCR positive who received high dose ganciclovir induction therapy. The half-life of decay for HCMV was calculated in each patient, assuming an exponential decline of virus, using pre and post induction therapy HCMV loads.

5.2.1 Frequent Sampling of HCMV Load During Induction Therapy

Five of the patients described above had agreed to participate in a more intensive study of HCMV load during induction therapy. Blood samples were taken whenever routine phlebotomy was performed (usually thrice weekly) and urine samples were collected as frequently as possible.

5.2.2 Correlation Between HCMV Load, UL97 Mutations and Viral Fitness

Three patients (two from this cohort and one more recently enrolled to this study and, therefore, not previously mentioned in this thesis) who became HCMV PCR positive on maintenance therapy and had mutations in UL97, had multiple sequential samples of blood and urine subjected to QCPCR measurements and analysis of their UL97 genotype using the point mutation assay described in section 4.8.

5.3 Results

5.3.1 Dynamics of HCMV Load During Induction Therapy

As presented in section 2.9.2 the majority of patients, who were HCMV PCR positive on entering the study, became PCR negative after 21 days of standard GCV induction therapy. This resulted in a mean reduction of HCMV load in blood of 2.8 $\log_{10}$ genomes/ml (see Figure 2.4). By using equation 1 the half-life for HCMV in the presence of ganciclovir was calculated for each patient:

Equation 1;

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

$$\text{slope of decline} = \frac{\ln [\text{HCMV ge/ml Day 0}] - \ln [\text{HCMV ge/ml Day 21}]}{21^*}$$

* or 14 for the two patients who received only 14 days induction therapy.

The median $t_{1/2}$ for HCMV in blood in the 33 patients who were PCR negative at day 21 was 2.48 days and in urine was 2.4 days (Table 5.1). If all 39 patients were included, ie including the 6 patients who did not have a complete "virological" response to induction therapy, the median $t_{1/2}$ for HCMV in blood and urine was slightly longer at 2.51 and 2.43 days respectively (Table 5.1).

These half-lives for HCMV in blood and urine provide an upper estimate of the half-life as many patients may have been PCR negative prior to day 21. In order to address this question five patients who agreed to undergo much more frequent sampling of blood and urine were analysed.

Table 5.1: Median and mean $t_{1/2}$ for HCMV (days) following ganciclovir induction therapy (IT) in all patients who were HCMV PCR positive at diagnosis of retinitis. The table has been divided according to whether patients became PCR negative after induction therapy.

	IT responders only (n=33)		**All patients (n=39)**	
$t_{1/2}$ for HCMV (days)	**Blood**	**Urine**	**Blood**	**Urine**
median	2.48	2.4	2.51	2.43
mean	2.56	2.31	3.02	2.55
range	1.33 - 4.85	1.83 - 2.94	1.33-10.05	1.83-7.25

5.3.2 Frequent Sampling of HCMV Load During Induction Therapy

Each of the five patients' changes in HCMV load in response to treatment is shown in Figures 5.1 and 5.2. Patients 1 to 3 all received ganciclovir induction therapy of 5mg/kg/bd for 21 days. In all three cases HCMV was undetectable in blood and urine by the end of induction therapy. Patients 4 & 5 were found to have asymptomatic, peripheral HCMV retinitis on ophthalmological screening. At day 14 both patients' retinitis was deemed stable by the ophthalmologist and they were commenced on oral ganciclovir maintenance therapy (1g tds) after only 14 days of ganciclovir induction therapy. Patient 4, who presented with a viral load in blood below the median of the cohort, responded very quickly to induction therapy and HCMV was undetectable in blood and urine by day 7. However patient 5, who had a viral load in blood of 6.61 $\log_{10}$ genomes/ml, failed to become HCMV negative in blood or urine after 14 days ganciclovir. Patient 5 had a first progression of retinitis at day 40 compared to day 75 for patient 4.

In all five cases it appeared that there were two different phases of reduction in HCMV load, implying the presence of a bi-phasic process of viral clearance involving two different half-lives. Therefore, rather than using equation 1 which assumes simple exponential decline in virus load, curve fitting algorithms based on the two phase exponential equation, equation 2, seemed to model the data in blood and urine more accurately:

Equation 2: $$y_0 + A1e^{-(x-x0/t1)} + A2e^{-(x-x0/t2)}$$

The estimated half -lives for HCMV decay in blood and urine for each of the five patients are shown in Table 5.2. This apparent two phase decay of HCMV may represent clearance of virus from two separate compartments. For example, the first more rapid decay may be due to clearance of free virus in the plasma, whereas the second and slower phase of decay may represent the clearance of virus from intracellular sites.

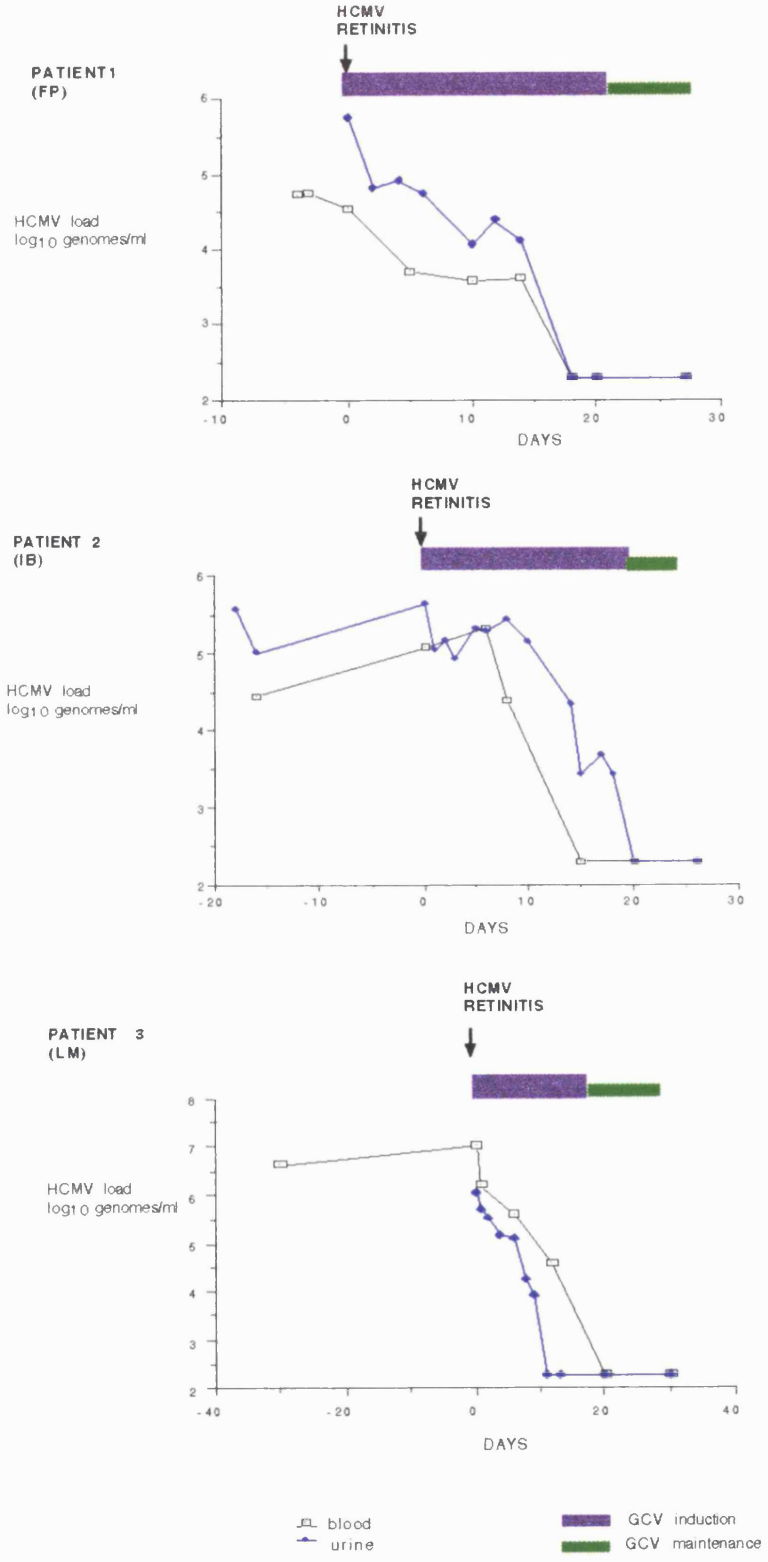

Figure 5.1: Changes in HCMV load (log_{10}genomes/ml) in blood and urine of patient 1 (FP), patient 2 (IB) and patient 3 (LM) during 21 days of ganciclovir induction therapy (5mg/kg/bd intravenously).

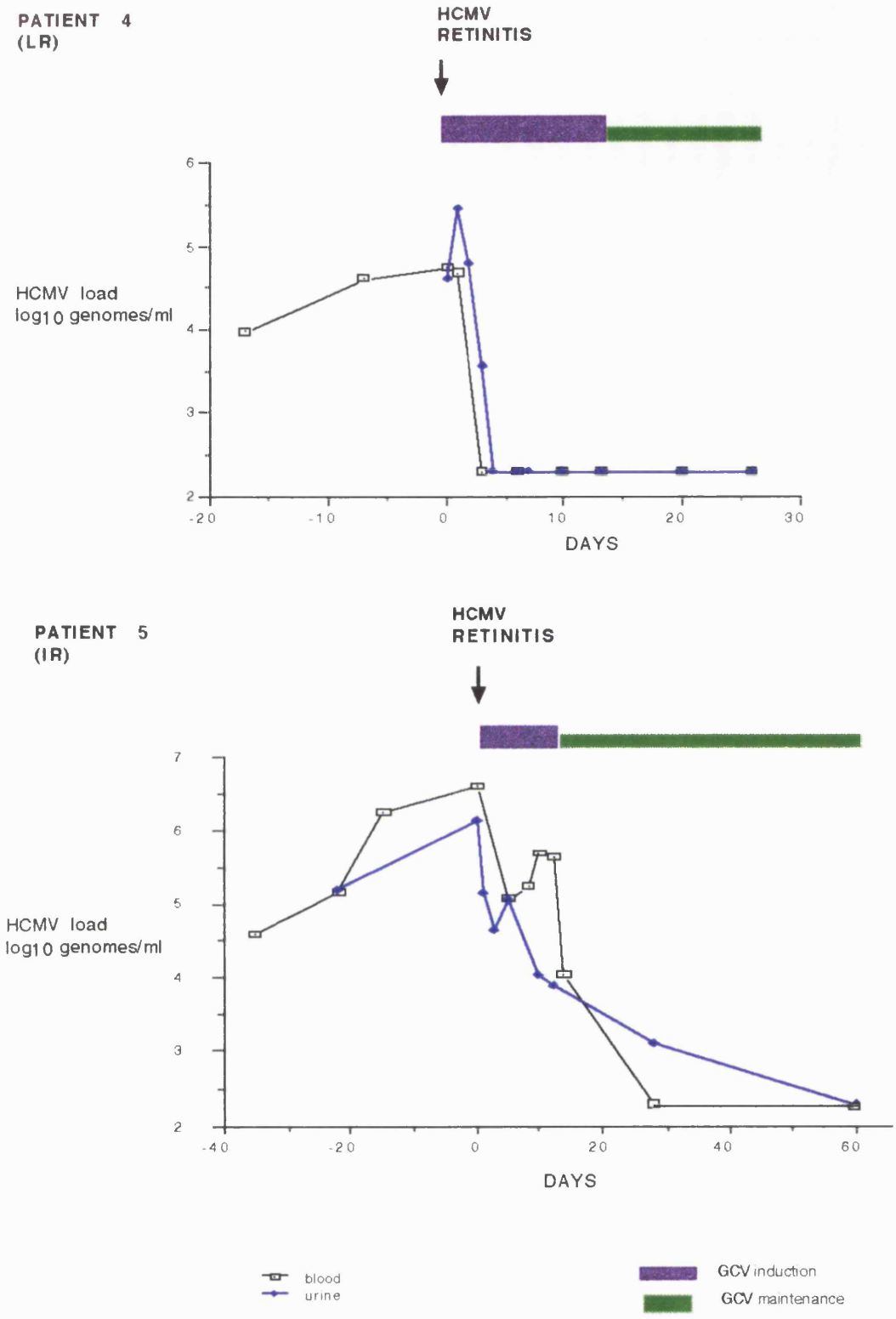

Figure 5.2: Changes in HCMV load (log_{10} genomes/ml) in blood and urine of patient 4 (LR) and patient 5 (IR) during 14 days ganciclovir induction therapy (5mg/kg/bd intravenously) and, thereafter, ganciclovir maintenance therapy (1g tds, po).

Table 5.2: Estimated half-life of HCMV (days) in blood and urine showing a bi-phasic response during ganciclovir induction therapy.

Patient ID	Sample	Half life (days)
1	Blood	t1 = 0.28 t2 = 5.0
	Urine	t1 = 0.28 t2 = 5.0
2	Blood	t1 = 0.25 t2 = 4.8
	Urine	t1 = 0.30 t2 = 7.5
3	Blood	t1 = 0.41 t2 = 2.78
	Urine	t1 = 0.46 t2 = 3.86
4	Blood	t1 = 0.40 t2 = N/A
	Urine	t1 = 0.40 t2 = 5.5
5	Blood	t1 = 0.32 t2 = 5.0
	Urine	t1 = 0.39 t2 = 5.1

5.4 Use of the Point Mutation Assay to Study Viral Fitness of UL97 Mutants

The PMA was used to study the evolution of both the UL97 L595 mutants (S595 and F595) and the M460I mutation in relation to each patients' clinical course and the anti-HCMV therapy they received. In all three cases the codon in question was wildtype at the onset of HCMV retinitis before any ganciclovir treatment was initiated. All three patients then experienced a period of HCMV PCR negativity and stability of their HCMV retinitis. In the case of the two patients with a mutation at codon 595, HCMV was detectable in blood several weeks before progression of their retinitis and codon 595 was mutant in the first HCMV positive sample. The third patient however, was HCMV PCR negative during his first two episodes of retinitis progression and did not become PCR positive until after 193 days of ganciclovir therapy.

5.4.1 Viral Fitness Associated with L595S Mutation in UL97

Patient 1 (RJ) developed a S595 mutation which was first identified with the resurgence of viraemia at day 100 of ganciclovir maintenance therapy (see Table 5.3). The viral load profile, percentage of mutant virus at codon 595 and clinical course of the patient are shown in Figure 5.3. At resurgence of viraemia, and throughout the study, the UL97 sequence at codons 460, 520 and 594 remained wildtype. The viral population remained mutant at codon 595 during continued exposure to ganciclovir. At the initiation of cidofovir treatment (day 279) the virus was 90.5% mutant at codon 595 (TCG). However, during the subsequent 151 days of cidofovir therapy, reversion of the 595 mutation to the wildtype TTG codon was observed (see Table 5.3).

Table 5.3: Percentage mutant virus (TCG, serine) at codon 595 as determined by point mutation assay, during treatment of HCMV retinitis with ganciclovir and cidofovir.

Patient 1 (RJ)

Time (days)	Status of retinitis	Treatment	% S595 mut
0	Newly diagnosed	IV GCV induction	8%
179	Stable	Oral GCV	94%
241	First progression	IV GCV re-induction	97%
248	Stable	IV GCV	96%
274	Second progression	IV GCV re-induction	95%
279	Still active	Initiated on CDV	90.5%
301	Stable	CDV	70%
329	Stable	CDV	61%
361	Stable	CDV	19%
375	Stable	CDV	3%
403	Stable	CDV	7%
430	Stable	CDV	8%

Died 12.4.96 of haemorrhagic pancreatitis ? HCMV related.

All urine samples from diagnosis to death were HCMV PCR negative.

IV GCV; intravenous ganciclovir 5mg/kg/bd

Oral GCV; oral ganciclovir 1g tds

CDV; cidofovir 5mg/kg intravenously weekly (induction) for two weeks then fortnightly (maintenance).

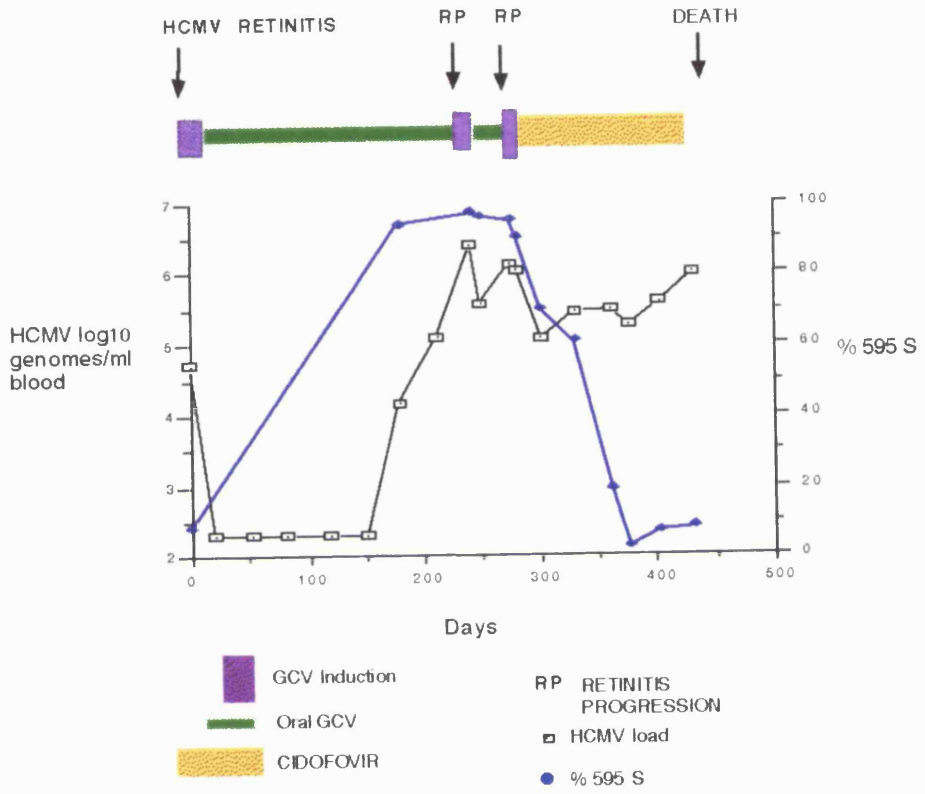

Figure 5.3: Alterations in HCMV load, 595 genotype and anti-HCMV therapy during 430 days of follow-up after a diagnosis of HCMV retinitis.

The fitness gain of the wildtype population was calculated using standard formulae for the effects of selection at a single locus in an asexual haploid population as previously used for HIV (Goudsmit *et al.* 1996). Assuming replication in continuous time, s, the selection coefficient (the fitness difference between mutant and wildtype) is given by the formula:

Equation 3: $$s = \frac{1}{t}\ln\left[\frac{q(t)p(0)}{p(t)q(0)}\right]$$

where q is a proportion of the more fit variant at time 0 and time t whilst p is the proportion of the less fit variant at time 0 and time t. The results of the PMA at the initiation of CDV therapy (day 279) gave the starting concentration of mutant (90.5%) and wildtype (9.5%) at initiation of CDV treatment whilst the numerical values for p[t] and q[t] can be derived from the PMA data at the appropriate times of sampling. Using the data at day 50 of CDV treatment (ie day 329 following diagnosis of retinitis, Table 5.3) where q[t] = 0.39 and p[t]= 0.61 with q[0] and p[0] set as 0.095 and 0.905 respectively then s is calculated as 0.0349, corresponding to a fitness loss of approximately 3.49% for the S595 virus. Since the ratio of p[0]:q[0] will influence the calculated s value, the relative loss of fitness was recalculated assuming a 5% and 1% prevalence of wildtype virus at initiation of CDV therapy. For these different initiation ratios of q[0] the relative fitness loss of the S595 mutant was 5% and 8.3% respectively. In order to ascertain which starting value of q[0] was most likely, the data generated by the PMA were superimposed on a series of computed curves for the re-population of the wildtype strains with time. These results are shown in Figure 5.4 and indicated that an initial q[0] between 0.01-0.05 resulted in the best correlation with the PMA data.

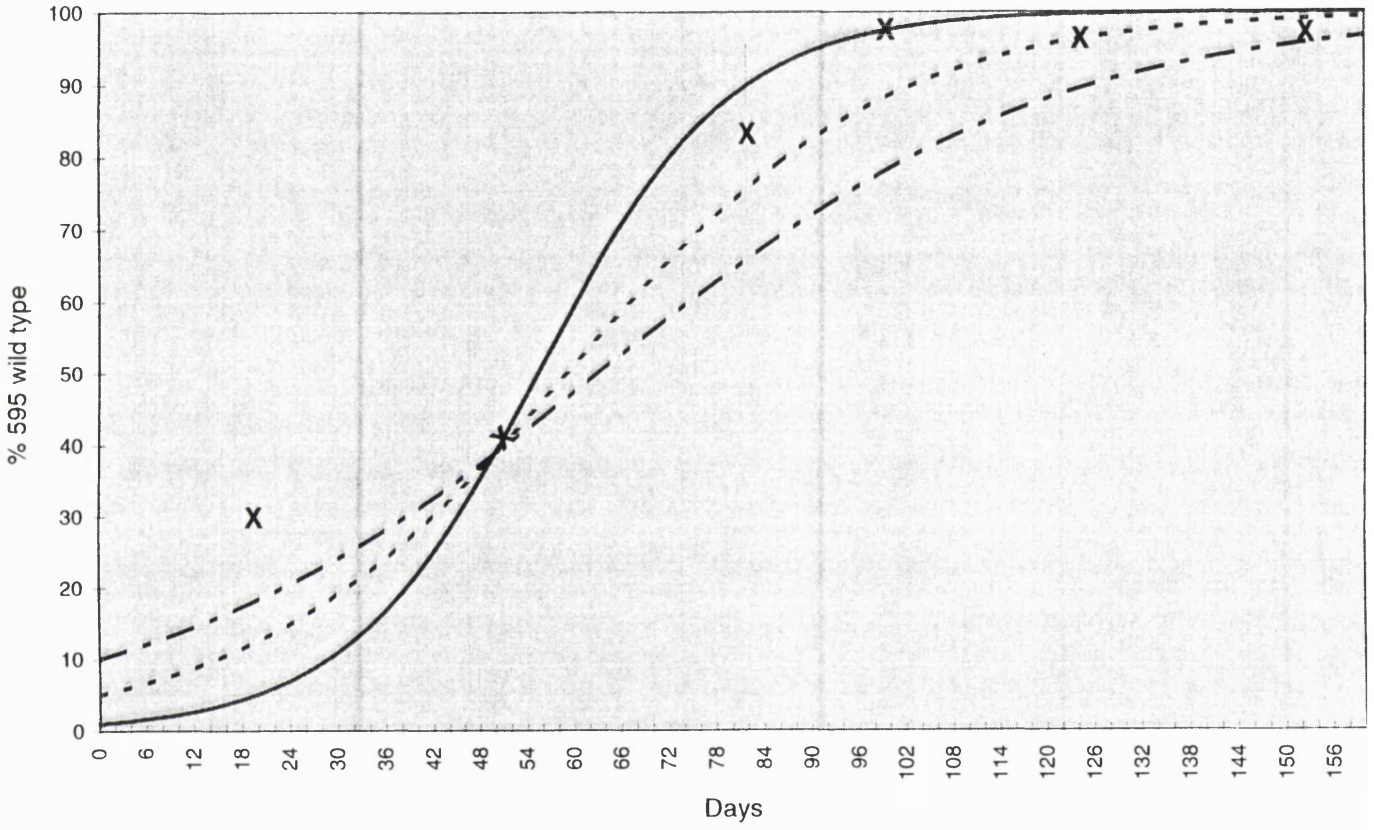

Figure 5.4: Simulation of the re-population rates of wildtype UL97 (codon 595), assuming a 1%, 5% or 10% population of wildtype virus at initiation of CDV, calculated using equation 2 and the PMA results at day 50 of CDV treatment. For comparison, the percentages of wildtype 595, generated by the PMA, are superimposed (x).

5.4.2 Viral Fitness Associated with L595F Mutation in UL97

Patient 2 (LM) was found to have a F595 mutation with resurgence of viraemia at day 93 of ganciclovir therapy and at day 127 suffered the first progression of his retinitis as shown in Table 5.4 and Figure 5.5. This mutation persisted throughout ganciclovir therapy and remained even after the patient had been started on foscarnet therapy to control his retinitis. During the first 308 days of treatment HCMV was not detected in urine. However, urine became HCMV PCR positive at day 322 and was wildtype at codon 595 despite still being mutant at codon 595 in the peripheral blood (Table 5.4). At this stage the patient, still on foscarnet maintenance therapy, went home to Norway for convalescence. Whilst in Norway he was changed to high dose ganciclovir therapy for three weeks following a diagnosis of HCMV oesophageal ulceration and then put back onto foscarnet maintenance therapy. Interestingly, on his return to this hospital, HCMV in his urine, which was initially wildtype at 595, had mutated to F595. HCMV in both blood and urine remained F595 mutant until the patients death at day 478.

Using equation 3 (see section 5.4.1) the fitness, s, for F595 over wildtype virus in the presence of ganciclovir was calculated in both blood and urine. Viral fitness in blood was calculated at day 93 where mutant virus was 79% and, with a pre-treatment level of F595 virus at 9%, the fitness of F595 was 0.039. Therefore, in the presence of ganciclovir and foscarnet, F595 mutant virus in blood was 3.9% more fit than wildtype virus. The fitness of F595 in urine was calculated at day 397, 75 days after urine had become PCR positive with a starting level of F595 of 4%. In urine the F595 mutant was calculated to be 6.7% more fit than S595 wildtype in the presence of ganciclovir and foscarnet.

Table 5.4: Percentage mutant virus (TTT, phenylalanine) at codon 595 as determined by point mutation assay, during treatment of HCMV retinitis with ganciclovir and foscarnet.

Patient 2 (LM)

Time (days)	Status of retinitis	Treatment	% F595 mut
0	Newly diagnosed	IV GCV induction	9% blood 5% urine
93	Stable	Oral GCV	79% blood
111	Stable	Oral GCV	95% blood
127	First progression	IV GCV re-induction	99% blood
134	Stable	IV GCV	99.1% blood
180	Stable	Oral GCV	97% blood
204	Second progression	IV GCV re-induction	99.6% blood
225	Stable	Oral GCV	97% blood
272	Third progression	Foscarnet induction	98% blood
308	Stable	Foscarnet maintenance	97% blood
322	Stable	Foscarnet maintenance	97% blood 4% urine
397	Retinitis Stable HCMV GI disease	Ganciclovir induction Foscarnet maintenance	97% blood 87% urine
438	Fourth progression	Intra-vitreal foscarnet	95% blood 90% urine
468	Stable	Intra-vitreal foscarnet	88% blood 92% urine

Died 18.11.96

IV GCV; intravenous ganciclovir 5mg/kg/bd

Oral GCV; oral ganciclovir 1g tds

Foscarnet induction; 90mg/kg bd daily

Foscarnet maintenance; 90mg/kg od daily

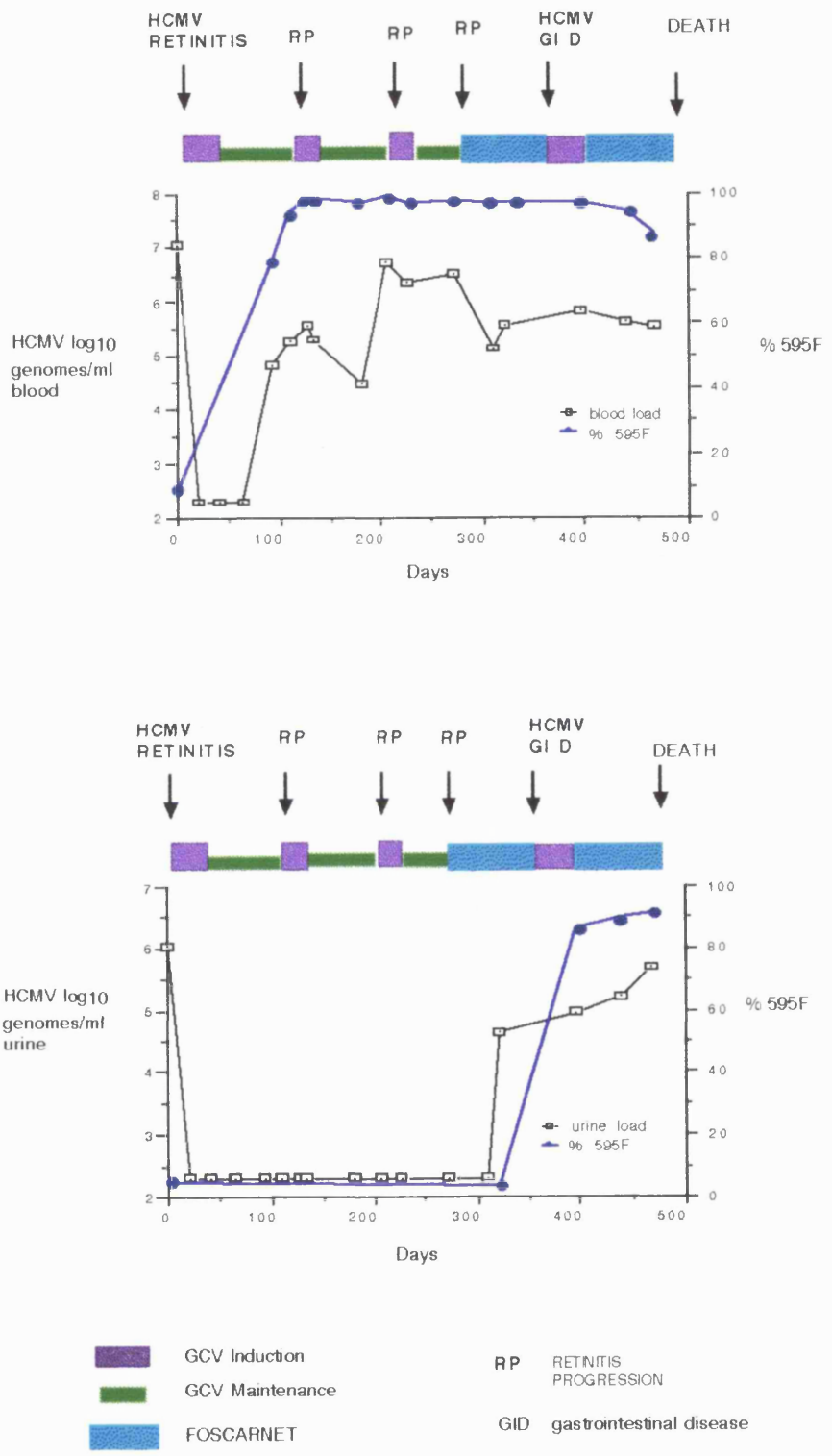

Figure 5.5: Alterations in HCMV load, 595 genotype and anti-HCMV therapy during 468 days of follow-up after a diagnosis of HCMV retinitis.

5.4.3 Viral Fitness Associated with M460I Mutation in UL97

Patient 3 (GS) followed a different pattern on maintenance therapy to the other two patients. He was PCR negative when he had his first two progressions of retinitis before becoming HCMV PCR positive at day 198 (see Table 5.5). At that time his retinitis was stable but was found, by the PMA, to have a mutation at codon 460, M460I and at 595, L595F. The percentage of I460 virus increased on ganciclovir whilst the percentage of F595 mutant stayed constant at 80%. Cidofovir was commenced at day 280 following a third progression of retinitis and by day 330 virus had become wildtype at both codons 460 and 595 (see Table 5.5).

In this patient HCMV was observed to change from mutant to wildtype during ganciclovir therapy and then revert to wildtype in the presence of cidofovir. Since the PMA indicated 100% wildtype virus at both codons pre-treatment, the fitness gain of I460 in comparison to M460 at days 198 and 260, assuming different levels of mutant prior to ganciclovir, was calculated:

%mut prior to GCV	I460 (d198)	I460 (d260)	F595
0.1%	s=0.031	s=0.033	s=0.041
0.05%	s=0.038	s=0.036	s=0.045
0.01%	s=0.043	s=0.042	s=0.053

Therefore, the I460 mutant virus was between 3.1% and 4.3% more fit than wildtype 460 in the presence of ganciclovir (see Figure 5.6 a). The same calculation can be done from days 198 to 280, when the level of F595 mutant virus remained constant (Table 5.5), in an attempt to take each mutation in isolation. Therefore, assuming the I460 mutant was in isolation, mutant virus was 5.6% more fit than wildtype.

However, the composition of the species was observed to change in the presence of cidofovir and both I460 and F595 mutants reverted to wildtype. Using the PMA data from day 320 (after 40 days of cidofovir) and, knowing from the PMA that there was 2% wildtype virus present at day 280, wildtype I460 was 12.8% less fit than M460 in the presence of cidofovir (see Figure 5.6 b). Using similar methods, the mutant F595 virus was calculated to be 3.6% less fit than wildtype 595 in the reversion of mutant virus to wildtype. For the reversion of this double mutant virus back to wildtype virus, it is difficult to estimate the contribution each mutant may have in isolation on viral fitness.

Table 5.5: Percentage mutant virus (ATT, inosine) at codon 460 as determined by point mutation assay, during treatment of HCMV retinitis with ganciclovir and cidofovir.

Patient 3 (GS)

Time(days)	Status of retinitis	Treatment	%I460mut	%F595mut
0	Newly diagnosed	IV GCV induction	wt	wt
77	First progression	IV GCV re-induction	PCR neg	neg
133	Second progression	IV GCV re-induction	PCR neg	neg
198	Stable	Oral GCV	32%	78%
260	Stable	Oral GCV	84%	79%
274	Third progression	Intravitreal foscarnet	93%	77%
280		CDV	98%	81%
296	Stable	CDV	98.5%	78%
301	Stable	CDV	98%	66%
320	Stable	CDV	22%	24%
330	Stable	CDV	4%	2%

Retinitis stable and patient well as of 3rd January 1997 (day 330).

IV GCV; intravenous ganciclovir 5mg/kg/bd

Oral GCV; oral ganciclovir 1g tds

CDV; cidofovir 5mg/kg intravenously weekly (induction) for two weeks then fortnightly (maintenance).

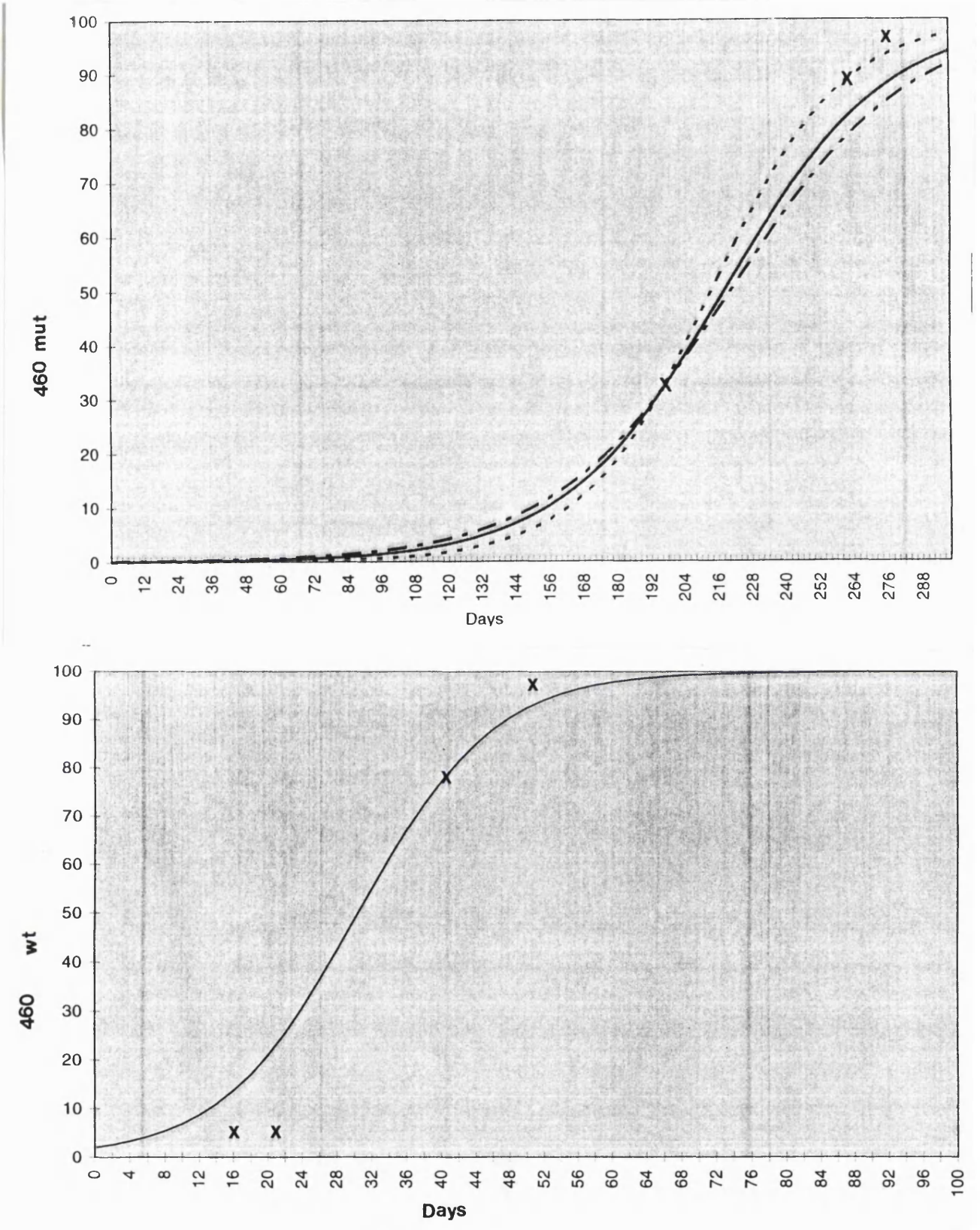

Figure 5.6: a)Calculated curves for re-population with mutant I460 virus in blood in the presence of ganciclovir therapy and b) for the re-population with wildtype M460 virus in the presence of cidofovir. For comparison, the percentages of mutant and wildtype 460 generated by the PMA, are superimposed (x).

5.5 Calculating the Generation Time of HCMV

Viral fitness can also be calculated using the assumption that two viral populations possess a constant fitness and grow in discrete non-overlapping generations. The proportion of the less fit population changes in time according to equation 4 where τ is the number of generations that have occurred in time t;

Equation 4:

$$p(t) = \frac{p(0)q(t)(fitness)^{\tau}}{q(0)}$$

Rearrangement of equation 4 to equation 5 allows the generation time of HCMV to be calculated if the relative fitness, s, of each virus is known;

Equation 5:

$$\tau = \frac{\log\left[\frac{p(t)q(0)}{p(0)q(t)}\right]}{\log(fitness)}$$

For example; by using the relative fitness for 595 in patient 1 at day 50 of CDV therapy, where s=0.961; p[t]=0.61, q[0]=0.095, p[0]=0.905 and q[t]=0.39, the number of generations which HCMV has passed through was calculated to be 52 from equation 5. Since this number of generations has passed in 50 days, the generation time of HCMV was calculated as 1.04 days.

Similar calculations for patient 2 resulted in a generation time of 1.02 days for HCMV in blood and 1.03 days in urine. The generation time for patient 3, both wildtype to mutant at day 198 and the reversion of mutant to wildtype at day 320, were also very similar at 1.02 and 1.07 days respectively. Therefore, in all three patients the generation time of HCMV was approximately 1 day.

5.6 Discussion

The viral generation time for HCMV is not known. Using crude calculations of the HCMV loads of all patients who received induction therapy in this study (using pre and post ganciclovir induction therapy measurements) the estimated half-life of decline in the presence of ganciclovir was approximately 2.5 days in blood and 2.43 days in urine. These calculations are probably under-estimates of half-life as many of the patients may have become HCMV negative some days before the end of induction therapy. Indeed, in a recent trial at the Royal Free Hospital of 14 vs 21 days induction therapy where patients can only be randomised to the study if they are HCMV PCR negative in blood at day 10, over 75% of patients receiving induction therapy have been eligible for the study. This would imply that the calculated half-life for HCMV in blood is probably significantly shorter than the 2.5 days calculated.

Using the results obtained from more frequent sampling in a much smaller number of patients, there appears to be a definite two phase decay of HCMV both in blood and urine with T1's of 0.32 and 0.39 days and T2's of 4.9 and 5.1 days respectively. The initial rapid decay may be due to the immediate inhibition of circulating virus, ie virus in plasma, that is mediated by drug alone. The second and slower decay may represent the clearance of cell associated virus which involves the recruitment of both cellular and humoral components of the hosts immune system to eliminate all infected cells. This second phase may be more sensitive to the continued deterioration in the hosts immune function and may explain why some patients do not become PCR negative after re-induction therapy even when virus is known to be wildtype.

The point mutation assay was used to analyse the relative fitness of different UL97 mutants in the presence of anti-HCMV therapies. In the presence of ganciclovir, HCMV in blood carrying the mutations F595 and/or I460 was 3.9% and 3.8% more fit than wildtype virus. F595 mutant virus in urine was 6.7% more fit than wildtype virus in urine. By changing anti-viral therapy and altering the selection pressure, mutant virus in patients 1 and 3 reverted to wildtype. In the presence of cidofovir the S595 and F595 mutants were 3.5-8.5% less fit than wildtype virus at codon 595 and the I460 mutant was 12.8% less fit than the M460 species. The co-existence of 460 and 595 mutations in patient 3 did not appear to affect the reversion of either mutation to wildtype although the 460 mutation appeared much less fit than the other mutations. The observed fitness of wildtype over mutant virus in this patient probably reflects the contribution of both the M460 and L595 strains to the fitness loss of the mutant virus. The true distribution of different mutations present on the same genome ie genetic linkage, requires further investigation with DNA sequencing.

In patient 2 HCMV remained mutant at codon 595, in the presence of ganciclovir and foscarnet, until his death suggesting the co-existence of a mutation in UL54 which has been described (Baldanti *et al.* 1996). The viral load in patients 1 and 3 remained high on cidofovir therapy despite the reversion to wildtype virus. This may be due to the presence of mutations in UL54 or may be due to the inability of cidofovir to effectively clear HCMV in the blood whilst being concentrated in the retina. Sequencing of UL54, which is currently underway, will help to answer these questions.

Up until the studies described in this chapter, an accurate assessment of the viral generation

time for HCMV has not been available. The results from the PMA were also used to calculate a generation time for HCMV. This was approximately 1 day. This rate is faster than that observed during *in vitro* propagation of HCMV strains and suggests that HCMV infection is a very dynamic process *in vivo*. The generation time derived is much lower than the half-life of virus in the blood of 2.4 days estimated from the whole cohorts response to induction therapy. This would imply that the half-life of HCMV during ganciclovir induction therapy must be much lower than calculated, closer to the T1 of approximately 0.5 days, otherwise HCMV would be seen to increase during the early stages of induction therapy. Further larger studies are needed to more accurately calculate both the generation time of HCMV and the half-life of HCMV in the presence of anti-viral therapy. Such a study, looking at the changes in HCMV load with frequent sampling of blood and urine in patients receiving oral ganciclovir pre-emptive therapy, is currently ongoing.

CHAPTER 6

BACULOVIRUS EXPRESSION OF UL97

6.1 Introduction

One approach to study the phenotypic significance of UL97 mutations in relation to HCMV sensitivity to ganciclovir, would be to culture HCMV *in vitro* for susceptibility assays. However, HCMV can be difficult to culture, especially in the presence of ganciclovir and strains with mutations in UL97 may be debilitated for growth. Therefore, growth *in vitro* may exert selective pressures so that the strain isolated may not be representative of the strain in the patient. For all of these reasons, I chose to use molecular methods to study variation in UL97. Genetic changes observed in UL97 were transferred into a system able to express the mutant protein whose biochemical characteristics could then be studied.

UL97 has been expressed successfully in *E.coli* and vaccinia virus systems. The seminal paper on the protein expression of UL97 involved the cloning of a truncated part of the UL97 ORF (UL97tr, Littler *et al* 1992) into the prokaryotic expression vector pGMT7. This UL97tr did not code for the first 326 amino acids but retained all the sequences that pertained to the putative catalytic domains of the protein kinases. pGMT7 relies on the bacteriophage T7 promoter to drive expression of the inserted sequence of interest. The plasmid containing the UL97tr, when transformed into *E.coli* BL21 cells, directed the expression of a protein of Mr 39,000 which was largely insoluble (Littler *et al* 1992). Littler *et al* confirmed the viral specificity of the recombinant protein by western blotting and its phenotypic function by its ability to phosphorylate ganciclovir (Littler *et al* 1992).

The whole of the UL97 ORF has been expressed in the eukaryotic vector p7.5K131, a vaccinia virus expression vector, both as wild type (derived from Ad169) and as a deletion

mutant (the laboratory strain 759rD100) (Metzger *et al* 1994). Recombinant UL97 vaccinia virus demonstrated a new phenotype that was ganciclovir sensitive. Moreover, incorporation of ^{14}C ganciclovir triphosphate was completely inhibited by guanosine suggesting that the latter may be a natural substrate for UL97.

The use of a eukaryotic expression vector allows the production of foreign proteins to a high yield within a eukaryotic environment. Apart from the vaccinia virus expression system, the other commonly used eukaryotic system involves baculoviruses, a family of large double-stranded DNA viruses that infect insects. Baculoviruses are named in accordance with their host of origin and are classified in relation to the type of occlusion structures that baculoviruses make during an infection (Bishop *et al* 1990). The baculoviruses that have been exploited as vectors are the baculovirus specific to the alfalfa looper moth, *Autographa californica* (AcNPV) and the silkworm virus *Bombyx mori* (BmNPV) (Emery 1991). During normal infection the viruses produce large numbers of intranuclear inclusions, or polyhedra, which consist of virions encased within crystals composed of a single protein of M_r 29,000, known as polyhedrin. Transcription of the polyhedrin gene is driven by a highly active promoter and therefore, because the polyhedrin gene product is not essential for viral replication, the polyhedrin gene can be replaced and its promoter used to drive expression of a foreign gene inserted downstream.

In order to generate a recombinant baculovirus the gene of interest must first be cloned into an appropriate transfer vector. All of the transfer vectors commercially available have *E.coli* origins of replication and an antibiotic resistance gene *in situ* allowing for easy selection of transformants. The choice of transfer vector also depends on whether the protein of interest is to be expressed as a fused or non-fused product. The expression of protein fused, for

example, to a sequence of six histidine residues, can be easily purified using nickel-agarose chromatography once the recombinant transfer vector has been co-transfected with full length baculovirus DNA into cultured insect cells. The two species of DNA, once internalised by the cell will recombine at the specific locus resulting in the insertion of the foreign ORF into the baculovirus DNA. Wild type baculovirus DNA (AcNPV) can be used but has a very low recombination frequency of <0.1%, necessitating the screening of large numbers of plaques to identify putative recombinants. The use of linearized wild type DNA increases the frequency of recombinant progeny to over 30%. Newer commercially available baculovirus DNA constructs have made use of lethal deletions to increase both frequency of recombination and ease of selection of recombinant phenotypes. Baculogold™ DNA (Pharmigen, UK) and Bac 'N' Blu™ DNA (Invitrogen, Holland) have had part of the essential 1629 ORF deleted. Since this deletion can only be repaired by recombination with a polyhedrin based transfer vector carrying ORF 1629, only replication competent virus should be generated by successful recombination. Bac 'N' Blu™ DNA also carries a mutant form of the β-galactosidase gene, so following co-transfection with a transfer vector carrying both the 1629 ORF and the β-galactosidase gene, recombinant plaques can be identified easily by their blue staining in the presence of X-gal. To enable internalisation of both types of DNA into the insect cells, their membranes must be made permeable to the baculovirus DNA. An efficient method is the use of cationic liposomes that form complexes with the DNA and facilitate interaction with the negatively charged insect cell membrane. Once transfer vector DNA and AcNPV DNA have been taken up into the cell recombination occurs.

This chapter describes the cloning and expression of the catalytic portion of UL97 into a baculovirus expression system as a method to produce sufficient protein to investigate the biochemical phenotype of clinical samples that have genotypic mutations in UL97.

6.2 Methods

6.2.1 Blunt-ended Ligation of UL97 into a Baculovirus Transfer Vector

UL97 DNA amplified by PCR from clinical samples or Ad169 was cloned into pUC18 as described in section 4.6. Particular pUC18 clones that were known to contain the UL97 mutations of interest and wild-type UL97 were re-sequenced using the M13 forward and reverse primers to identify the orientation of the UL97 fragment. This enabled cloning into the appropriate ribosomal reading frame in the chosen vector. Sequencing of the clones was carried out as described in section 4.7, apart from the use of 7-deaza-dGTP labelling mix diluted 1 in 10 with SDW to allow the reading of bases very close to the primer.

The baculovirus expression vector chosen was the plasmid BlueBacHis vector (pBBH, Invitrogen, UK.), due to the easy identification of recombinant plaques and purification of expressed protein. This vector (as shown in Figure 6.1) has a polyhedrin promoter that controls expression of the gene of interest and protein is expressed as a histidine tagged fusion protein. Three different vectors were available (pBBH A, B and C) each having the gene insertion point in a different reading frame. For UL97 inserts cloned into pUC18 in the 5' to 3' orientation, BBH B was the vector of choice and for inserts in the 3' to 5' orientation, BBH A.

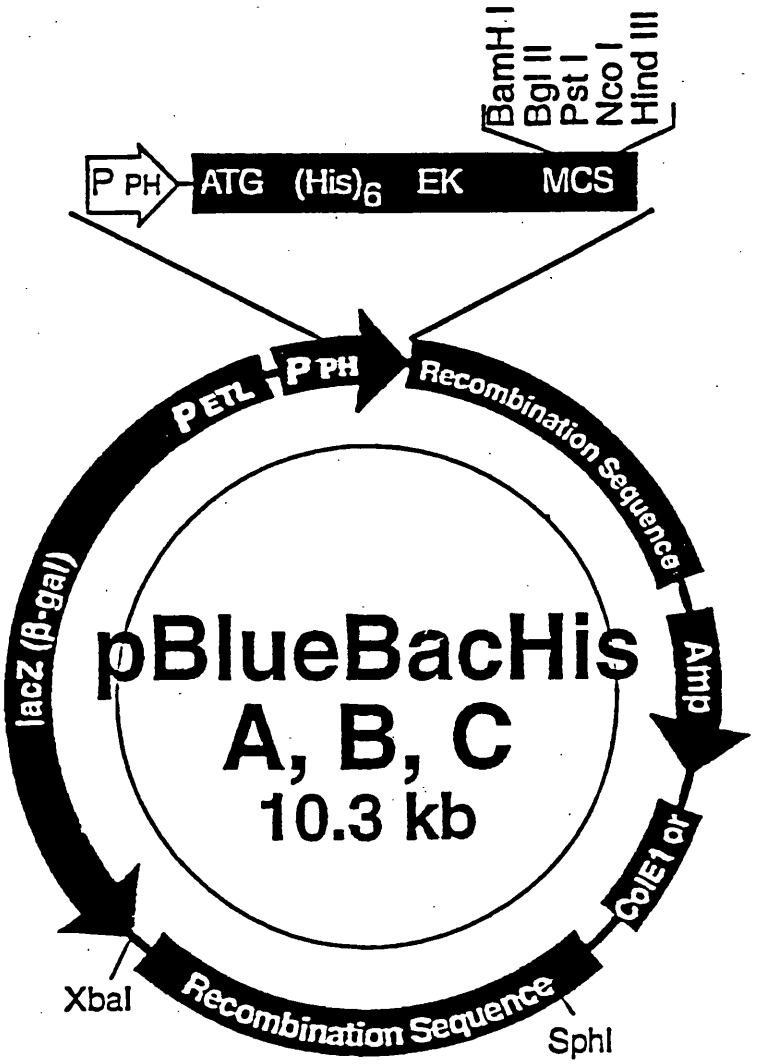

Figure 6.1: Basic structure of the pBlueBacHis (A, B and C) transfer vector. Illustrating the multiple cloning site (MCS) for the insertion of the gene of interest downstream of the polyhedrin promoter (P PH) and the six histidine residues $(His)_6$ to aid protein purification. The *lacZ* gene, allowing colour screening of recombinants, is also shown.

6.2.2 Dephosphorylation of Transfer Vector DNA.

10μg of the appropriate vector was linearised by digestion with 2 units of *BamH1* in a total reaction volume of 20μl and incubated at 37^{0}C for 1 hour. The linearised DNA was then excised and purified from a low melting point agarose gel using the "Gene Clean" kit as described in section 4.5.1. To prevent recircularisation of vector DNA and allow blunt-end ligation with the 5' phosphorylated UL97 fragment from pUC18, pBBH A/B was dephosphorylated. The linearised pBBH vector was treated with 1 unit of calf intestine alkaline phosphatase (Boehringer Mannheim), 5μl 10x dephosphorylation buffer (500mM Tris-HCl, pH 8.5, 1mM EDTA), adjusted to a final volume of 50μl with distilled water and incubated at 37°C for 30 min. The enzyme was inactivated by the addition of 5mM EDTA and incubation at 75°C for 10 min. The vector DNA was purified by phenol extraction, followed by ethanol precipitation, as detailed in section 3.2.3, and resuspended in 10μl of SDW.

6.2.3 Enzyme Digestion of pUC18 Containing UL97 Sequence

The UL97 pUC18 clone of interest from each clinical sample and Ad169 was subjected to double enzyme digestion with *EcoRI* and *HindIII*, in the presence of 10x buffer, to excise the UL97 sequence. The recessed ends of the UL97 fragment were filled using the Klenow fragment of DNA polymerase I in the presence of 10x MSK buffer and 2mM dNTPs as described in section 4.6.2. The blunt-ended and purified UL97 DNA fragment was then re-analysed on a 1% agarose gel in comparison to a λ *HindIII/EcoR1* DNA marker.

6.2.4 Ligation of UL97 into BlueBacHis

125ng of dephosphorylated BlueBacHis A/B vector and 40ng of UL97 insert were incubated overnight at 16°C in the presence of 1.25 units of T4 ligase, 1mM ATP and 10x ligation buffer as described in section 4.6.2. A control ligation reaction was prepared with linearised dephosphorylated BlueBacHis vector and all ligation reagents but without insert DNA. The ligations were used to transform competent JM109 *E.coli* as described in section 4.6.4. The subsequent *E.coli* transformants were plated onto LB agar plates containing 50μg/ml of ampicillin.

6.3 Cloning of "Sticky-ended" UL97 into BlueBacHis

6.3.1 Generation of UL97 Amplicons with Restriction Sites

In initial results I had been unable to successfully clone UL97 into the baculovirus vector by blunt ended ligation. In order to improve the efficiency of transformation I decided to clone UL97 directly into pBBH from pUC18 using compatible enzyme digestion resulting in a 'sticky-ended' ligation. Firstly, UL97 DNA was amplified from a clinical sample carrying the L595F mutation and from Ad169 using phosphorylated primers. The primer pair was designed to incorporate a *BamHI* restriction site in the sense primer (Bac1) and a *HindIII* restriction site in the anti-sense primer (Bac2) thus ensuring that the UL97 insert was cloned into pUC18 in the correct orientation. The primer sequences are as follows with the restriction endonuclease sites shown in italics;

Bac1; 5' ***G↓GATCC***GTGGACATGAGCGACGAGAGCTAC

Bac2 ; 5' ***A↓AGCTT***CCGCGTCCCGGTTACTCGGGGAAC

A full optimisation of the PCR, as a single round PCR, was carried out with regards to both primer and $MgCl_2$ concentrations and annealing temperature as detailed in section 4.4. The resultant 1160bp UL97 amplicons were excised and purified from a low melting point agarose gel, Klenow-filled and cloned into pUC18, that had been digested with *SmaI* and dephosphorylated, as described in section 4.6.2. The ligations were used to transform competent JM109 *E.coli* as described in section 4.6.4. The subsequent *E.coli* transformants were plated onto LB agar plates containing 50μg/ml of ampicillin. Multiple colonies were picked and selected for screening as described in section 4.6.5. Successful ligations were confirmed by the presence of the 1160 bp insert following double enzyme digestion of DNA with *BamHI* and *HindIII* and electrophoresis on a 1% agarose gel.

Recombinant clones of pUC18 containing the UL97 gene were subjected to a full double enzyme digestion using *BamHI* and *HindIII* in 10x enzyme dilution buffer B. Following electrophoresis on a 1% low melting point agarose gel, the UL97 insert was excised and purified as described in section 4.5.1.

6.3.2 Ligation of Sticky-ended UL97 into BlueBacHis

In order to keep the 1160 bp UL97 product from pUC18 in the correct open reading frame, the pBBH vector A was used throughout as the transfer vector. BBH A was linearised by restriction enzyme digestion with *BamHI* and *HindIII* in 10x enzyme dilution buffer B with a one hour incubation at 37°C. The digested products were electrophoresed on a 1% low melting point agarose gel, excised and purified. 100ng of BBH A was ligated to 11ng of UL97 (as a 1:1 molar ratio) in the presence of 1.25 units of T4 ligase (Northumbria), 1mM ATP (Amersham) in ligation buffer in a total volume of 10μl. A

control ligation was prepared with linearised BBH A vector and all ligation reagents but without insert DNA. The ligation reactions were incubated overnight at 16°C. 10µl of each ligation reaction was then used to transform 100µl of competent *E.coli* (JM109) cells as described in section 4.6.4. The resultant transformants were plated out onto LB agar plates containing ampicillin (50µg/ml) and colonies were selected for plasmid isolation and screening for UL97 using *BamHI* and *HindIII* digestion. Eight colonies that contained the appropriately sized insert after the above digestion were subjected to sequence analysis. Clones were sequenced, as described in section 4.7, using forward and reverse baculovirus primers to ascertain whether the UL97 gene had been successfully inserted (pBluUL97) and was in the correct reading frame. The primer sequences were as follows;

Bac F; TTTACTGTTTTCGTAACAGTTTTG

Bac R; CAACAACGCACAGAATCTAGC

The internal primers SC2 and SQ2 (described in section 4.5.2 and illustrated in Figure 4.2) were used to sequence a small fragment of UL97 around the 595 region to confirm the presence of wildtype L595 (pBlu595L) or mutant F595 (pBlu595F). The point mutation assay was also used to screen multiple clones for the presence of mutant or wildtype 595 (see section 4.8).

6.3.3 Large Scale Preparation of Purified pBluUL97

One crucial factor in the transfection efficiency of baculovirus insect cells with DNA and transfer vector is the quality and purification of the DNA. Commercially available "maxi-prep" kits, using anion-exchange resins to bind plasmid DNA, produce DNA as pure

as that from caesium chloride gradients. Therefore, DNA was prepared from two clones of pBlu595L and pBlu595F known to be in the correct reading frame by plasmid sequencing. Firstly, each clone was grown up overnight at 37°C in 5ml LB broth containing ampicillin. This 5ml culture was transferred into 95ml LB broth containing ampicillin (50μg/ml) and incubated in a shaker at 37°C for 6 hours. A culture volume of 1ml was expected to yield 300-500μ DNA using the Qiagen "Maxi-Prep" protocol.

The cell culture was centrifuged at 5000g at 4°C for 10 min in a IEC-7000 centrifuge. The bacterial pellet was resuspended in 10ml of buffer P1 (50mM Tris-HCl, pH8.0, 10mM EDTA) and then mixed gently with 10ml buffer P2 (200mM NaOH, 1% SDS) and incubated at room temperature for 5 min. The lysate was neutralised with 10ml of chilled buffer P3 (3M potassium acetate, pH 6.5), placed on ice for 20 min and then centrifuged at 4°C, 5000g for 30 min. The resulting supernatant was filtered and passed down a Qiagen tip-500 (pre equilibrated with 10ml buffer QBT; 750mM NaCl, 50mM MOPS, pH 7.0, 15% ethanol and 0.15% Triton X-100). After the supernatant passed down the tip by gravity flow, the resin tip was washed twice with 30ml wash buffer (1M NaCl, 50mM MOPS, pH 7, 15% ethanol) and the DNA was eluted in 15ml elution buffer (1.25M NaCl, 50mM Tris-HCl, pH 8.5, 15% ethanol). The DNA was precipitated by the addition of 10.5ml isopropanol and 30min centrifugation at 5000g 4°C. The resulting DNA pellet was washed twice with 70% ethanol, air dried and the DNA dissolved in 300μl TE.

6.4 Co-transfection of Insect Cells

Baculovirus DNA and the baculovirus transfer vectors pBlu595L and pBlu595F were co-transfected into insect cells. Homologous sequences between the viral DNA and the

transfer vector should allow recombination resulting in replication-competent circular baculovirus DNA.

6.4.1 Insect Cell Culture

One of the most important aspects for a successful transfection, and all other aspects of baculovirus work, is the quality of the insect cells used. Sf21 cells (Invitrogen) were used for all experiments in this chapter. The cells were maintained in complete TC-100 medium (Gibco BRL) with supplements (penicillin 100u/ml and streptomycin 100mg/ml, Gibco) and 10% fetal bovine serum in 75cm^2 tissue culture flats (Costar) and incubated at 28°C. The cell monolayers were checked frequently and the cells passaged when confluent. For the purpose of transfection and plaque assays Sf21 cells were harvested at sub-confluence and seeded in complete TC-100 in 35mm diameter dishes at a concentration of 1 x 10^6 cells per dish. All insect cell culture and baculovirus DNA work was carried out under sterile conditions in a Class II cabinet and in a separate laboratory from all other HCMV UL97 work.

6.4.2 Co-transfection

In this transfection pBlu595L and pBlu595F were mixed together with either Baculogold or Bac-N-Blue DNA and Insectin™ liposomes in the presence of TC-100 medium (without supplements to avoid the proteins in the serum from interacting with the liposomes). The following components were added into a 1.5ml sterile Eppendorf tube;

pBlu595L and pBlu595F	4μg
Baculogold DNA (0.1μg/μl)	1μg (10μl)
or	
Bac-N-Blue DNA (0.1μg/μl)	1μg (10μl)
TC-100-serum free	1ml
Insectin liposomes (Invitrogen) (always added last)	20μl

The transfection mixture was vortexed vigorously for at least 10 seconds and then left to incubate at room temperature for 15 minutes. In the meantime, Sf21 cells seeded in 35mm dishes (Costar) were washed with serum free TC-100. The entire transfection mixture was then gently added separately to each dish and left to incubate at room temperature for 4 hours. Following the 4 hour incubation period, 1ml of complete TC-100 was added to each dish and incubated at 28°C in a humidified chamber.

The cells were inspected on a daily basis. At between 4 and 5 days signs of infection became evident, by the visualisation of polyhedra and the supernatant was harvested by pipetting off the medium followed by centrifuging at 1000g for 5 min. The supernatant, containing virus, was transferred into a sterile 1.5ml Eppendorf tube and kept at 4°C whilst the cell fraction was stored at -70°C. The remaining cells were re-fed with ml of fresh complete TC-100 and incubated at 28°C for a further 48 hours. At this stage, if the recombination was successful, signs of late infection such as cessation of growth, detachment of the cells from the monolayer and the ballooning of cells were observed. If these signs of late infection were present, the harvested supernatant was plaque purified according to the procedure in the following section.

6.4.3 Plaque Assay of Co-transfection Supernatant

The purpose of the plaque assay is to purify the desired recombinant virus in the

supernatant away from wild type virus and other aberrant recombinations. Plaque purification was performed by infecting insect cell monolayers with serial dilutions of the transfection supernatant obtained in section 6.4.2 and isolating plaques of infection under an agarose overlay.

35mm dishes were seeded with Sf21 cells at a concentration of 1 x 10^6 cells/dish and incubated overnight at 28°C to achieve 50% confluency . The co-transfection stock from section 6.4.2 was made into serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} with TC-100 complete medium. The cells were infected with 100µl of each dilution in duplicate with two negative controls consisting of 100µl of complete media only. The viral stock was allowed to adsorb to the monolayer for one hour with gentle rocking of the plates every 15 min and then removed. 3% w/v agarose/SDW (molecular grade low melting temperature type VII, Sigma) was melted, cooled to below 55°C, and diluted to a final concentration of 1.5% using an equal volume of TC-100 complete medium. 2ml of the 1.5% agarose was immediately used to overlay the cells. The agarose was left to set for 20 min before 1ml of TC-100 complete medium was added to each dish and the dishes placed in a sterile humidified container at 28°C. The dishes were left for up to 6 days in the incubator and were observed every day for signs of plaque formation or confluency of uninfected controls.

6.4.4 Screening for Recombinant Plaques

The use of Baculogold and Bac-N-Blue DNA facilitates the screening of recombinants because they both carry the *lacZ* gene and upon recombination with pBluUL97 the *lacZ* gene is repaired (see Figure 6.2) and the recombinant plaques will be stained blue if a chromogenic substrate is included in the staining procedure. The sequence

of cellular events in the monolayer leading to plaque formation is shown in Figure 6.3. To stain the cells the medium was removed and 1ml of TC-100 complete medium containing 15µl of X-gal (50mg/ml) was added to each plate and left to incubate at 28°C for 3 hours. 1ml of PBS containing neutral red (0.025% v/v, Sigma) was then added to the plates and left for 2 hours. All medium was then carefully removed, the plates were blotted on tissue paper and placed upside-down in a darkened container and left overnight to destain. After 24 hours, blue recombinant plaques could easily be identified against the red background compared to the white plaques of wild type virus and aberrant recombinations. Eight blue plaques were picked by sucking out the agarose plug using a fine sterile glass micro-pipette. Each plaque was placed into 1ml of TC-100 complete medium and vortexed vigorously for 10 seconds to release the recombinant virus from the agarose. 100µl of each plaque/medium dilution was added to a 35mm plate containing Sf21 cells as described in section 6.4.3.

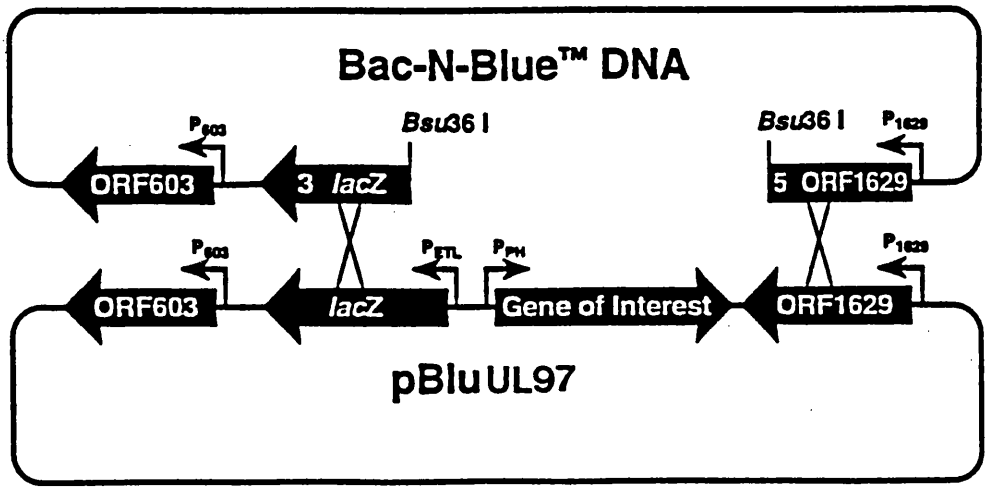

Figure 6.2: Recombination events between pBluUL97 and Bac-N-Blue DNA. Recombination occurs between the *lacZ* and ORF1629 sequences, forming blue, recombinant plaques on medium containing X-gal.

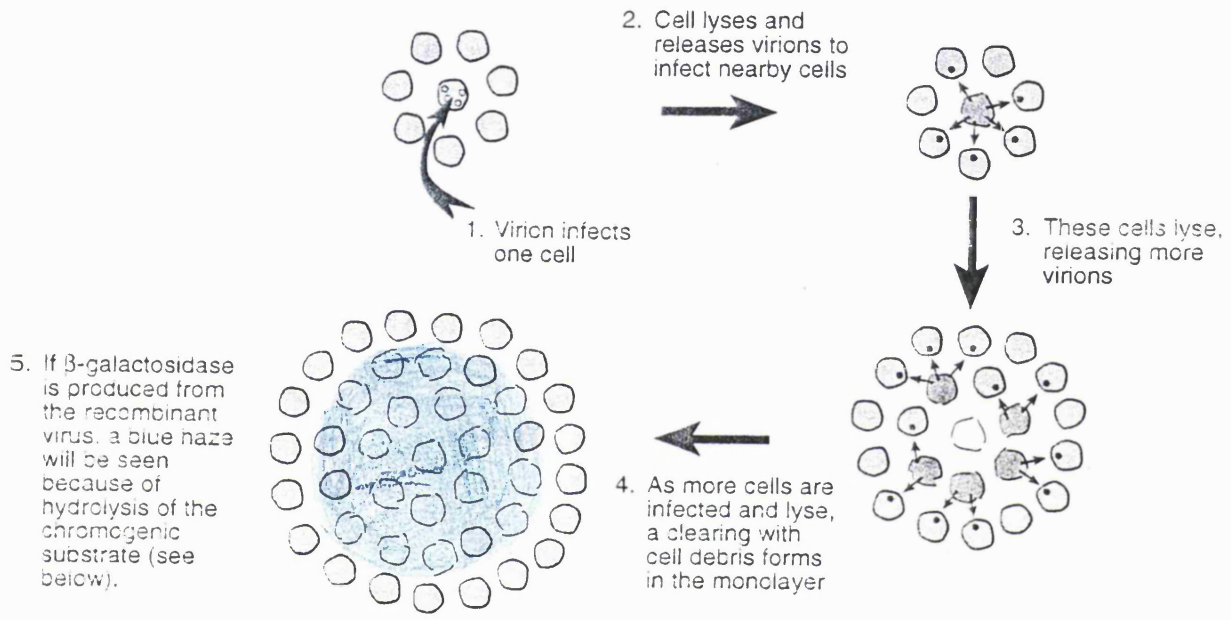

Figure 6.3: Schematic illustration of the cellular events that lead to plaque formation in an insect monolayer.

After 5 days incubation at 28°C the cells were resuspended into the medium and centrifuged at 13000g in a microcentrifuge for 3 min. The supernatant was removed and stored at 4°C whilst the cells were subjected to DNA extraction using the Puregene™ (Gentra systems, Inc.) extraction kit as follows. The cells were lysed by adding 600µl Cell Lysis solution and pipetting vigorously to lyse the cells. 3µl of RNase A Solution was added to the cell lysate and the sample incubated at 37°C for 60 minutes. After being allowed to cool to room temperature, 200µl of Protein Precipitation solution was added to the RNase A-treated cell lysate followed by centrifugation at 13000g for 3 min. The precipitated proteins formed a tight white pellet and the supernatant containing the DNA was removed and transferred into a clean 1.5ml Eppendorf tube containing 600µl isopropanol. After gentle inversion to precipitate the DNA and centrifugation at 13000g for 1 min the DNA was visible as a small pellet which was washed with 70% ethanol and left to air dry. The DNA was rehydrated overnight in 100µl DNA Hydration Solution and subsequently stored at -70°C .

6.4.5 PCR Analysis of Recombinant Plaques

The DNA extracted from the cell monolayer in section 6.4.4 was subjected to PCR analysis to screen for the presence of the UL97 insert in the putative recombinant virus. The recombinant baculovirus forward and reverse primers used were provided by Invitrogen. The primers were designed to flank the polyhedrin locus and are therefore compatible with all polyhedrin based transfer vectors. The forward primer binds 5' to the polyhedrin gene from nucleotide position -44 and the reverse primer binds 3' to the polyhedrin gene at position +79,. resulting in a wildtype Bac-N-Blue DNA product of 839 bp or a recombinant BluUL97 595F product of 1895 bp. The PCR reaction was set up as detailed in the

Invitrogen manual using 5µl of viral DNA in the presence of 100ng of each primer, 5µl 10x NH4 buffer (described in section 2.4), 2.5mM $MgCl_2$, 25mM dNTP's and *Taq* polymerase made up to a final volume of 50µl with SDW. Each reaction was overlaid with mineral oil and placed in a thermocycler programmed to the following parameters; a denaturation step at 94°C for 4 min for 1 cycle, then 30 cycles of 94°C for 1 min, annealing at a temperature of 55°C for 2 min and a primer extension step at 72°C for 3 min. The final cycle included a 7 min primer extension step at 72°C. 10µl of each PCR reaction was analysed on a 1% agarose gel in the presence of a λ *HindIII/EcoR1* DNA marker.

Supernatant from the plaques found to contain the UL97 insert was passaged in duplicate for a second time through Sf21 monolayers in a $25cm^2$ flask. After 2 days, half of the flasks were harvested for indirect immunofluoresence whilst the other flasks were maintained for 6 days and the supernatant harvested and stored at 4°C. This supernatant was then passaged in a $75cm^2$ flask as above. This P3 (virus passaged three times) stock was used to assay for protein expression.

6.4.6 Indirect Immunofluorescence of Infected Cells for UL97 Recombinants

Indirect immunofluorescence was performed using UL97 antiserum kindly donated by Dr Detlef Michel (Michel *et al* 1996). For indirect immunofluorescence the cells were gently harvested, washed twice in 500µl sterile phosphate buffered saline (PBS) and resuspended in 200µl PBS. 15µl of resuspended cells were pipetted onto a single well of a 12 well poly-tetra fluoro-ethylene coated slide (PTFE, Hendley-Essex, UK). Cells from each recombinant virus culture were tested in duplicate and there were two negative controls consisting of uninfected cells per slide. The slides were left to dry for 1 hour before being

fixed in ice-cold acetone and placed at -70°C for 10 min. The slides were then allowed to dry in a laminar air flow cabinet at room temperature and stored at -20°C. 10µl of UL97 antiserum (dilution factor 1:100 in PBS) was added to each well and the slides were placed in a humidified chamber at 37°C for 40 min. The slides were then washed with 1% bovine serum albumin (w/v, Sigma) in PBS. The secondary antibody layer consisted of 10µl fluorescein isothiocynate-conjugated (FITC) goat anti-rabbit IgG $F(ab')_2$ at a dilution of 1:20 (British Biosciences). The slides were again incubated in a humidified chamber at 37°C for 40 minutes before being washed in 1% BSA in PBS and then finally once in PBS alone. The slides were left to dry at room temperature before being mounted with a glass cover slip with the addition of "Citifluor"(UKC, Canterbury) to preserve the fluorescence. The slides were examined under a Zeiss immunofluorescence microscope and photographed with a Nikon camera using 1600 ASA film.

6.5 Visualising Proteins using SDS-Polyacrylamide Gel Electrophoresis

The P3 supernatant from section 6.4.5 was used to infect Sf21 cells seeded in 35mm dishes at a density of 1.5 x 10^6 cells/ml and incubated for 2 days. Three 35m dishes containing *lacZ*, wild type and negative controls were also prepared. After 2 days the cells were harvested, washed in PBS and then resuspended in 50µl sterile PBS and 50µl radioimmunoprecipitation (RIPA) buffer (1% Triton X-100 v/v, 1% sodium deoxycholate (w/v), 150mM NaCl, 50mM Tris-HCl pH 7.4, 10mM EDTA and 1% SDS (w/v)). Prior to loading the samples onto a SDS polyacrylamide gel, 100µl 2x sample buffer (2.3% SDS (w/v), 10% glycerol (v/v) 5% 2-mercaptoethanol, 62.5mM Tris-HCl pH 6.8 and 0.01% w/v bromophenol blue) with freshly added DTT was added to the suspension and the samples

were heated to 94°C for 10 min. In the meantime, two 10% (w/v) SDS polyacrylamide resolving gels were prepared as follows; 3.3ml of 30% polyacrylamide was mixed with 4ml SDW, 2.5ml 1.5M Tris, pH 8.8 and 100µl 10% SDS. The gel was polymerised with 100µl 10% APS and 6µl TEMED and poured between two clean glass plates as described in section 2.5.2. Approximately 5ml of the gel mixture was poured covering up to 75% of the gel plates, which was overlayed with butanol saturated with water. Once the resolving gel had set, the butanol was washed off and a stacking gel was poured on top and the gel combs inserted. The stacking gel was made of 1ml of 30% polyacrylamide, 6.3ml SDW, 2.5ml 0.5M Tris, pH 6.8 and 100µl 10% SDS and polymerised with 100µl 10% APS and 20µl TEMED. Once the stacking gel had set, 15µl of each sample (run in duplicate in adjacent wells) was loaded into each well. For each gel, molecular weight markers ("Rainbow" marker, MW 14,300-200,000 daltons, Amersham) were added to one sample well. The gel was subjected to electrophoresis at 200V for 70 minutes until the loading dye had reached the bottom of the gel.

6.5.1 Staining SDS-polyacrylamide Gels with Coomassie Brilliant Blue

The polypeptides separated by SDS-PAGE electrophoresis were fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue. One of the SDS-PAGE gels from section 6.5 was placed in 5 gel volumes of Coomassie Brilliant Blue stain, 0.25% w/v Coomassie Brilliant Blue R250 in fixative (45:45:10 methanol/SDW/glacial acetic acid) and left on a slowly rotating platform overnight. The gel was then destained in 5 gel volumes of fixative alone on a rocking platform for a minimum of 24 hours. The gel was then stored, prior to photography, in water in a sealed container.

6.5.2 Transfer of Proteins From SDS-polyacrylamide Gel to A Solid Support (Western Blotting)

The other SDS-PAGE gel from section 6.5 was rinsed in transfer buffer (39mM glycine, 48mM Tris base, 0.0375% (w/v) SDS, pH 9.6). Meanwhile six sheets of 3M filter paper were cut to the size of the gel, soaked in transfer buffer and then compressed onto the positive carbon plate of a semi-dry blotter (Pharmacia) by gently rolling a sterile glass pipette over the surface to remove any trapped air. A piece of Hybond-C Extra nitrocellulose membrane (0.45μm pore size, Amersham) was cut to the size of the gel, soaked firstly in methanol and then in the transfer buffer. The rinsed SDS gel was placed on the six sheets of 3M paper on the blotter and the nitrocellulose membrane carefully placed on top of the gel ensuring the whole gel was covered. A further 6 sheets of 3M filter paper soaked in transfer buffer were placed over the membrane and compressed to remove any air bubbles. The negatively charged carbon plate was placed on the surface and a current of 2.50 mA/cm^2 was applied across the blot for one hour.

After one hour the gel was carefully removed and placed into Coomassie Brilliant Blue stain to verify that the proteins had migrated from the gel to the membrane. The nitrocellulose membrane was washed twice in TBS buffer (10mM Tris-HCl, pH7.5, 150mM NaCl) for 10 minutes each wash and then left for one hour at room temperature in blocking buffer (3% BSA in TBS), to reduce non-specific binding, and then washed twice for 10 minutes with TBS-Tween/Triton buffer (20mM Tris-HCl, pH7.5, 500mM NaCl, 0.05% Tween 20, 0.2% Triton X-100 (Sigma)) and once with TBS buffer. The membrane was then incubated with the primary antibody, UL97 antiserum (1:100), in 1% blocking buffer (1% BSA in PBS) at room temperature for one hour. The membrane was washed twice with TBS-Tween/Triton

buffer and once with TBS buffer before incubation at room temperature for one hour with alkaline phosphatase conjugated goat anti-rabbit IgG (dilution 1 in 1000).

The membrane was then washed x 4 for 10 min in TBS-Tween/Triton and placed in staining solution (0.075M Tris-acetate ph 9.6, 0.005% (w/v) 4-nitro-blue-tetrazolium chloride, 0.00250% w/v 5-bromo-4- chloro-3-indoxyl -phosphate-p toludine, 0.2M $MgCl_2$) for 5-7 min after which it was washed in SDW, left to air dry and then photographed as soon as possible to prevent fading of the colour.

6.6 Results

6.6.1 Ligation of Blunt Ended UL97 into pBBH

Blunt ended ligation was initially used to clone the UL97 mutations that had been identified in pUC18 clones from different patient samples. This method allowed the direct excision of the gene of interest from pUC18 into a baculovirus transfer vector and therefore, assess the phenotypic function of each mutation that had been identified. However, multiple attempts at cloning several different UL97 mutants into pBBH were unsuccessful. There may have been several reasons for these unsuccessful ligations. One disadvantage of blunt ended ligation is that the ligation efficiency is lower than for sticky ended ligation. The second reason involves the dephosphorylation of BlueBacHis vector, where large amounts of DNA can be lost in the course of the procedure and failure of complete dephosphorylation allows cut vector to recircularise. Although pBBH was linearised and dephosphorylated and the UL97 insert cut out of pUC18 (see Figures 6.4 a & b respectively), all subsequent transformations failed to yield recombinant pBluUL97. Control ligations of linearised, dephosphorylated pBBH, without vector DNA, always yielded many colonies. This suggests that dephosphorylation of the vector was not complete. Screening of the large number of colonies using a plasmid preparation assay was a very time consuming process. Perhaps the use of other screening methods such as colony hybridisation would have been more efficient. However, as time was limited, the decision was taken to use a sticky-ended ligation procedure.

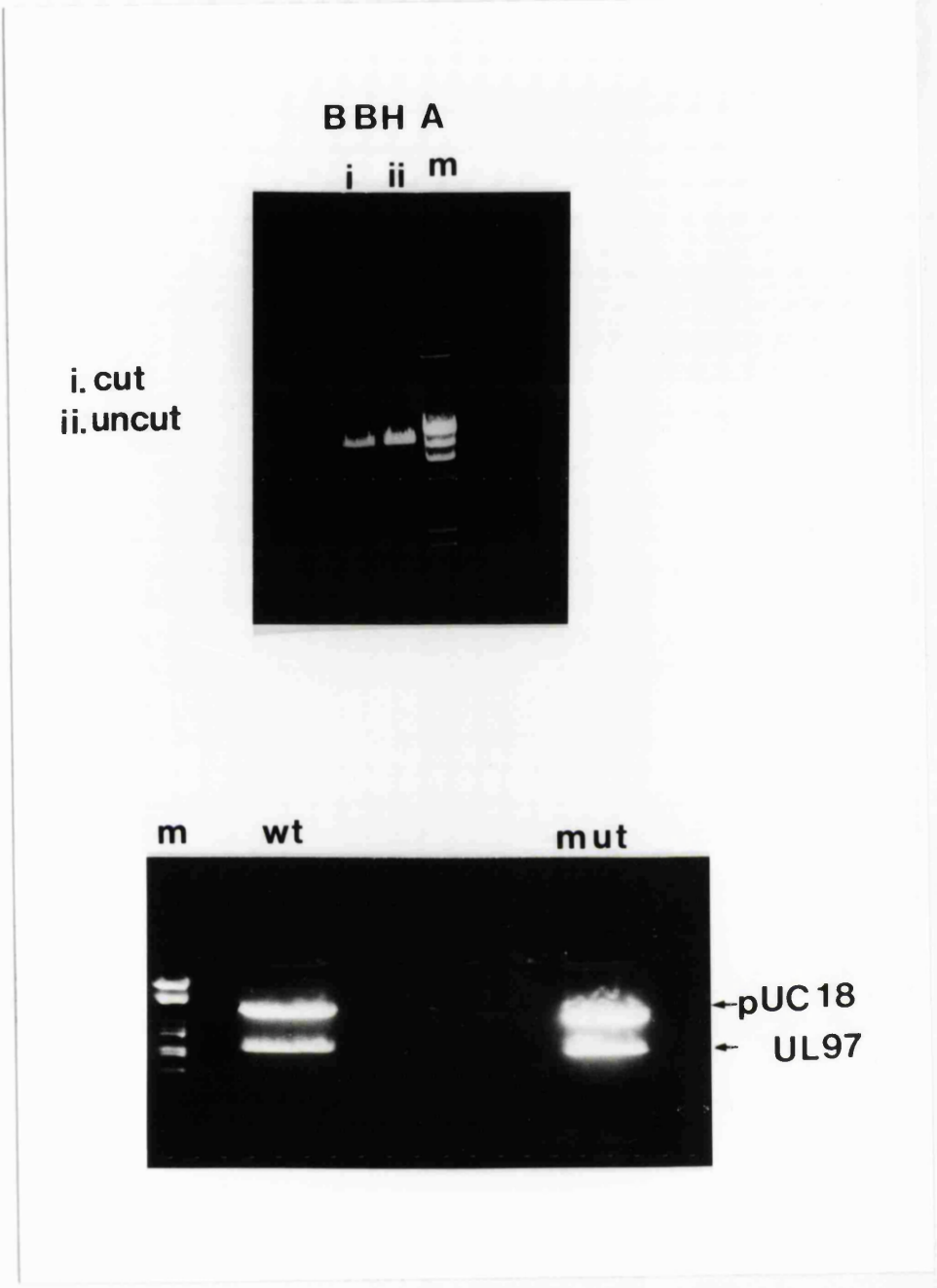

Figure 6.4: a) the linearisation of pBlueBacHis A using *BamHI* showing circular (ii, uncut) and linearised (i, cut) pBBH in comparison to a λ *HindIII/EcoR1* DNA marker (m); and b) the excision of wildtype (wt) and mutant (mut) UL97 DNA from pUC18 using *HindIII/EcoR1* in comparison to a λ *HindIII/EcoR1* DNA marker (m).

6.6.2 Sticky Ended Ligation of UL97 into pBBH A

The initial disadvantage of using this method was that the UL97 mutations already cloned into pUC18 were unsuitable as the *BamHI* and *HindIII* digestion sites in pUC18 did not flank the UL97 insert. I therefore, decided to focus on one patient with the F595 UL97 mutation who had progressive HCMV retinitis but whose HCMV could not be cultured for phenotypic analysis. The first step was to amplify UL97 using phosphorylated primers that introduced a *BamHI* and *HindIII* digestion site into a UL97 fragment of 1160 bp. The PCR was optimised using DNA extracted from patient samples. The optimised conditions included the use of 2mM $MgCl_2$ and an annealing temperature of 58°C in a single round PCR. One of the most critical components of the optimised PCR was found to be the primer concentration where 400ng of each primer was necessary for optimal UL97 amplification as shown in Figure 6.5.

UL97 fragments carrying the mutant F595 and wildtype L595 were successfully cloned by blunt ended ligation into *SmaI* digested pUC18 and then, using *BamHI* and *HindIII* double enzyme digestion, cloned directly into BlueBacHisA at the first attempt. Positive colonies were screened using conventional dideoxy sequencing methods with commercially available baculovirus primers to confirm the correct reading frame of each clone. Figure 6.6 shows the sequence of pBlu595F with the reconstruction of the *BamHI* site. To confirm the presence of either the mutant or wildtype genotype of the pBluUL97 clone, the point mutation assay was used to screen a large number of clones and subsequently, internal primers flanking the 595 codon were used to sequence the region (Figure 6.7). Figure 6.8 compares the UL97 DNA product from small scale (mini-prep) and large scale (maxi-prep) plasmid preparations.

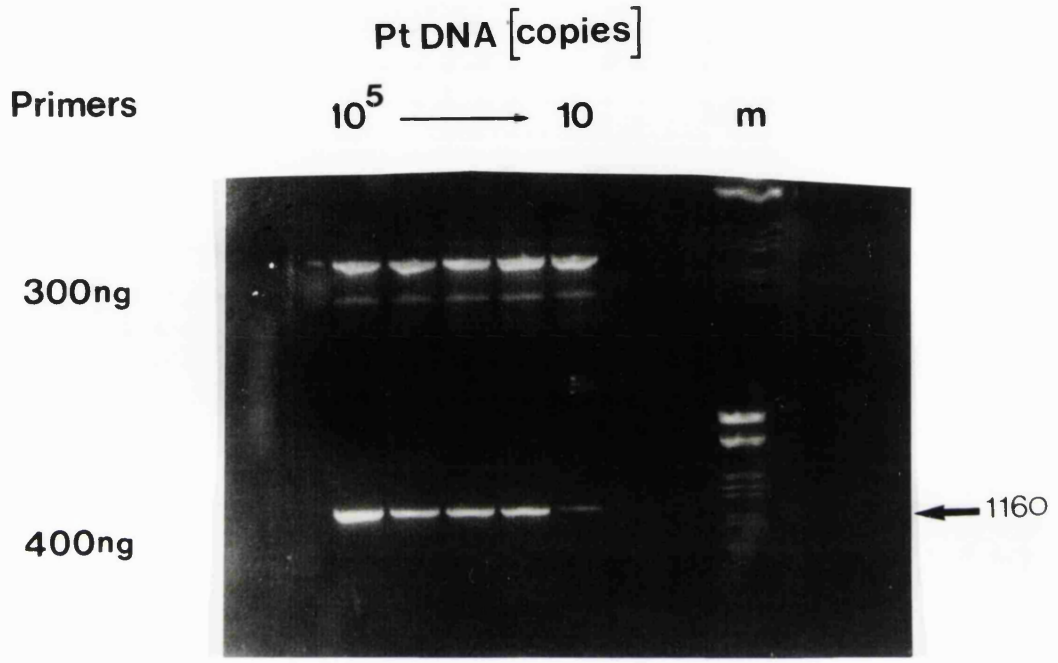

Figure 6.5: Titration of the concentration of the Bac1 & 2 primers at different copy numbers of patient DNA, showing the increased sensitivity and specificity of the primers when used at the higher concentration of 400ng in comparison to a λ *HindIII/EcoR1* DNA marker (m).

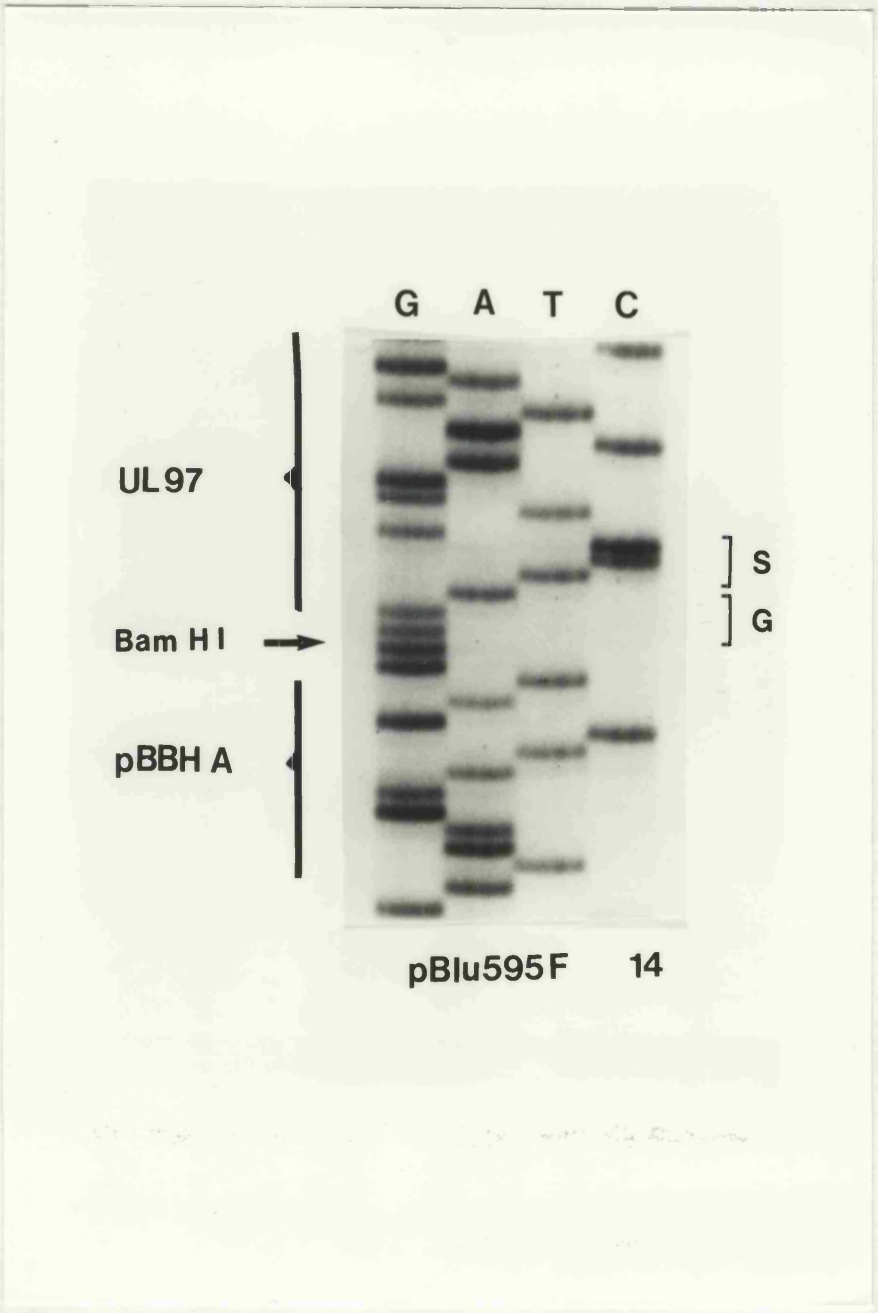

Figure 6.6: Autoradiograph of pBluUL97 (pBlu595F clone 14) in the correct ORF as shown by the correct sequence of amino acids that have been marked. The restoration of the *BamHI* site can be seen.

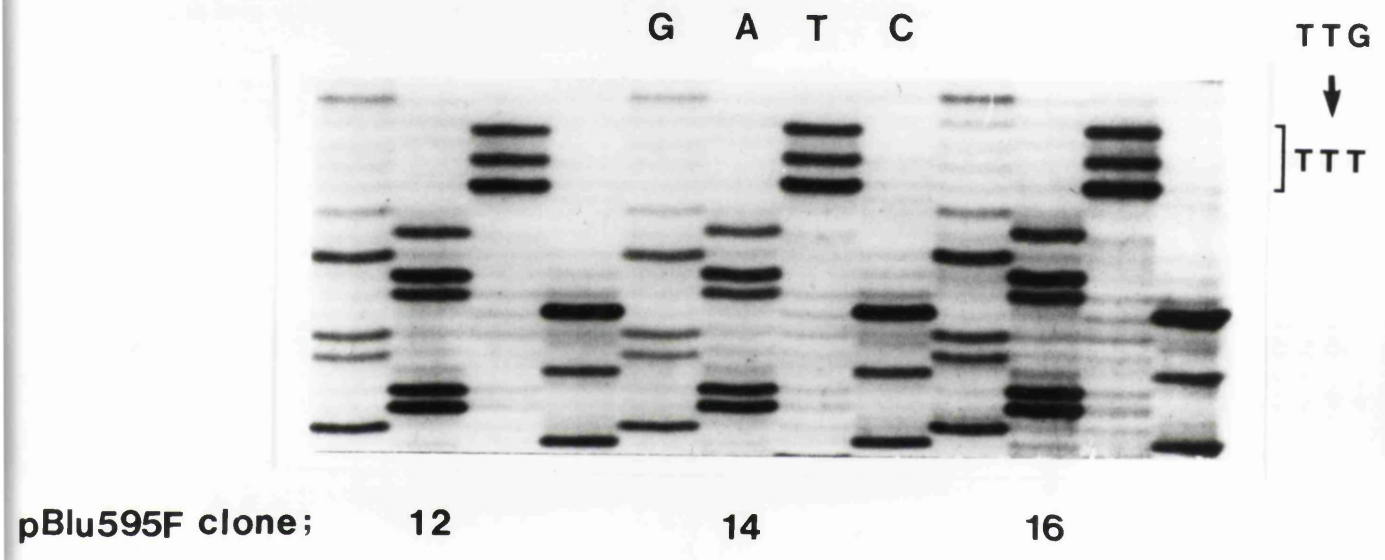

Figure 6.7: Internal oligonucleotide primers were used to confirm sequence of the pBluUL97 clones. All the clones sequenced here carried the F595 genotype.

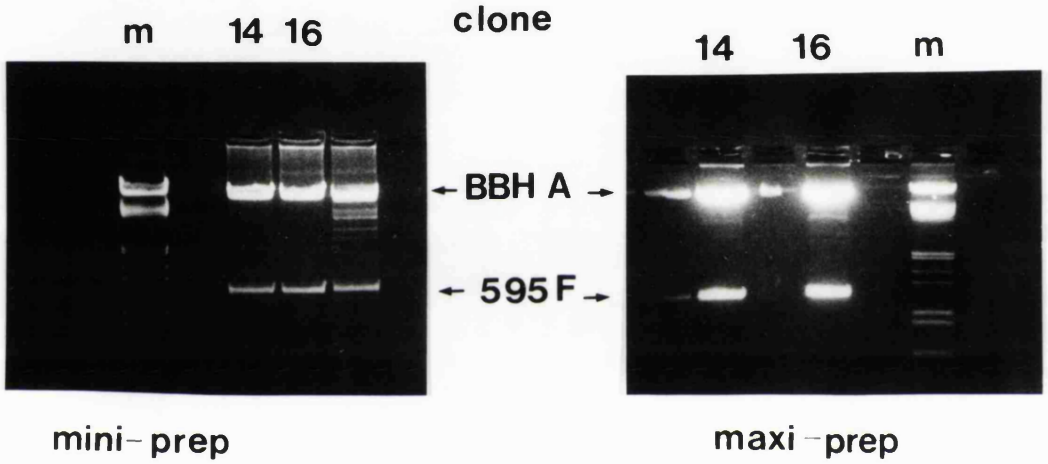

Figure 6.8: Clones were screened using DNA "mini-prep" assays. A much larger quantity of more purified pBlu595F DNA was then produced for co-transfection using a "maxi-prep"procedure. The size of the UL97 insert was checked in comparison to a λ *HindIII/EcoR1* DNA marker (m).

6.7 Co-transfection of pBlu595F with Baculogold/Bac-N-Blue DNA

Initially the transfection had to be repeated several times before achieving any positive plaques. The co-transfection was eventually successful using both Baculogold and Bac-N-Blue DNA. The quality of the baculovirus DNA and the liposomes were crucial for the success of the transfection. The transfection was so successful in the first instance that the initial plaque assays at 10^0, 10^{-1} and 10^{-2} were too concentrated resulting in the whole monolayer staining blue with X-gal. The plaque assay was therefore repeated using the transfection supernatant diluted to 10^{-2}, 10^{-3} and 10^{-4}. At the latter two dilutions blue plaques could be identified after staining with X-gal and counter-staining with neutral red. Figure 6.9 shows the presence of both blue, recombinant plaques and white wildtype plaques from the plaque assay of pBlu595F. The blue plaques were selected for screening.

6.7.1 Screening of Recombinant Plaques for BluUL97 595F

Eight blue plaques with Baculogold DNA and four blue plaques with Bac-N-Blue DNA were picked, the DNA was extracted and subjected to PCR analysis. The primers used were supplied by Invitrogen and resulted in either a 839bp fragment with wildtype Bac-N-Blue DNA or a 1895bp fragment with recombinant BluUL97 595F/Bac-N-Blue DNA (see Figure 6.10). Plaque pick 4 was the only pure recombinant virus as plaques 2 and 3 were also contaminated with wildtype virus. Therefore, plaque 4 (Blue 4,595F) of BluUL97 595F/Bac-N-Blue DNA was grown up to a high titre stock. All eight BluUL97 595F/Baculogold plaques were found to be recombinant. Plaque 7 (Gold 7,595F) gave the strongest after PCR and western blotting (data not shown) and was therefore, used to grow up to a high titre stock.

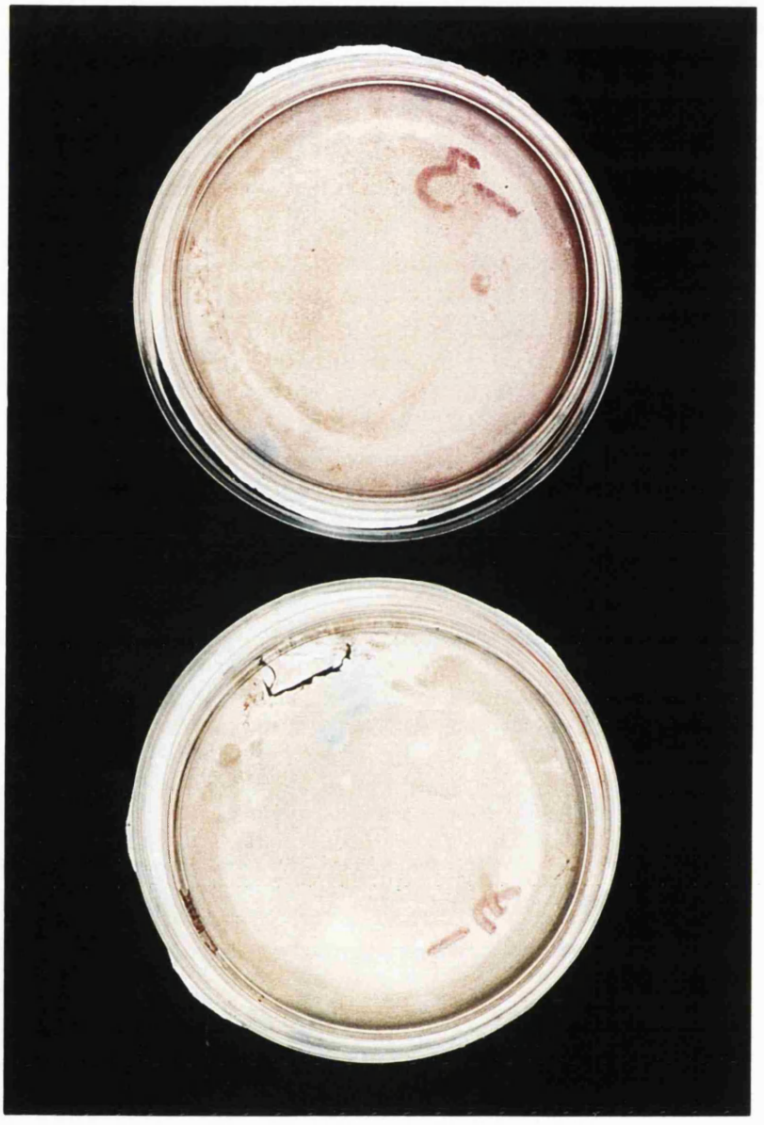

Figure 6.9: Plaque assay of pBlu595F/Bac-N-Blue DNA stained with X-gal and counterstained with neutral red. The blue plaques seen were picked for conformation of recombination. White plaques of wildtype virus can also be seen.

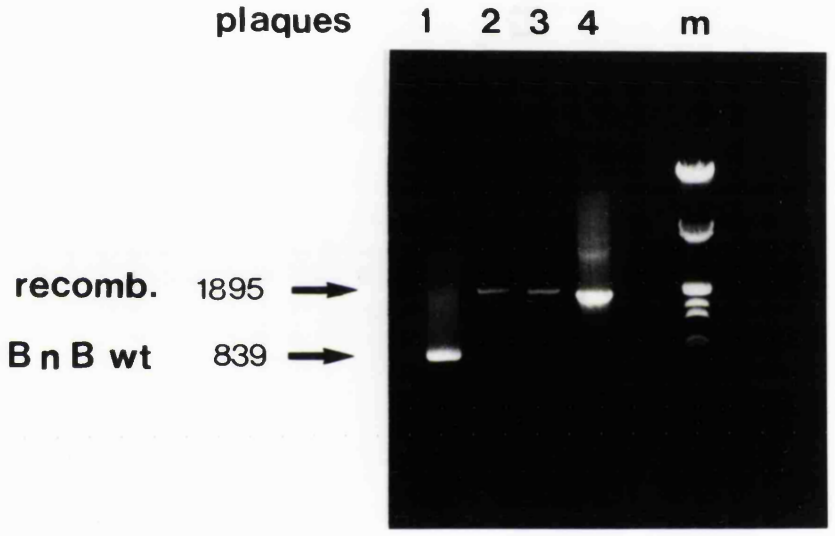

Figure 6.10: PCR screening of recombinant plaques of pBlu595F/Bac-N-Blue DNA in comparison to a λ *HindIII/EcoR1* DNA marker (m). Plaque 4 was recombinant pBlu595F only, whereas plaques 2 and 3 were contaminated with wildtype DNA. Plaque 1 is wildtype DNA only.

6.7.2 Indirect Immunofluorescence of BluUL97 595F for UL97

Both BluUL97595F stocks were used to infect insect cells and 48 hours following infection, were fixed and analysed for UL97 expression by indirect immunofluorescence. Both Blue 4,595F and Gold 7,595F gave an intense green granular peri-nuclear staining (see Figures 6.11 a&c respectively). Although the background staining on the negative cells was high, the characteristic granular peri-nuclear staining was absent (see Figures 6.11 b).

6.7.3 Visualisation of Proteins using SDS-Polyacrylamide Gel Electrophoresis

On staining the SDS-PAGE with Coomassie blue a distinct band was seen in the Gold 7,595F duplicate lanes running between the Mr 46,000 and Mr 30,000 bands of the rainbow marker as seen in Figure 6.12. The estimated size of the UL97 protein is Mr 46,000 Multiple bands were seen in both Blue 4,595F lanes that were also present faintly in the *lacZ* control and wildtype control although not in the negative control. It was therefore necessary to proceed to western blotting to confirm the presence of the protein.

Contrary to the results of the Coomassie stained gel, a very strong distinct protein band was seen in the two Blue 4,595F lanes and to a much lesser extent in the Gold 7,595F lanes (Figure 6.13). This band was of the appropriate size for the BluUL97 protein and was not seen in any of the control lanes.

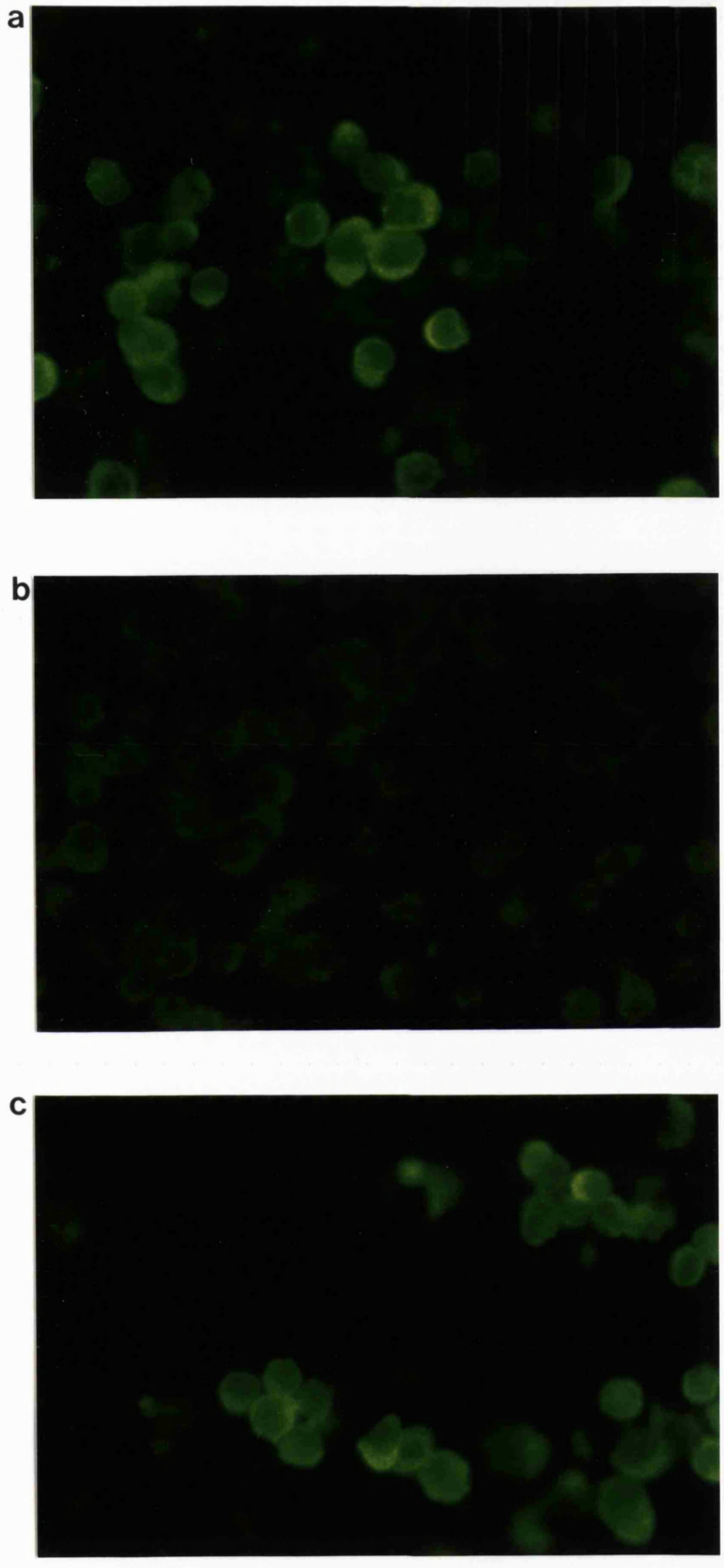

Figure 6.11: Indirect immunofluorescence of UL97 expressed by recombinant baculoviruses using a) Bac N Blu DNA and c) Baculogold DNA. 6.11 b) is uninfected cells only.

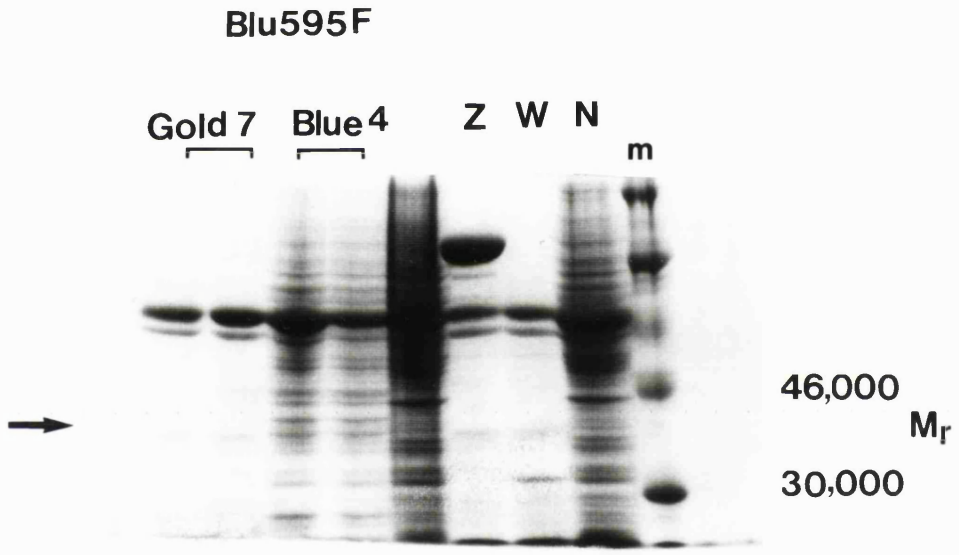

Figure 6.12: Coomassie brilliant blue staining of SDS-PAGE of BluUL97 infected Sf21 cells. The plaques Blue 4,595F and Gold 7,595F were run in duplicate alongside wildtype baculovirus DNA (W), a negative control (N) and a lacZ control (Z) all in the presence of a rainbow marker.

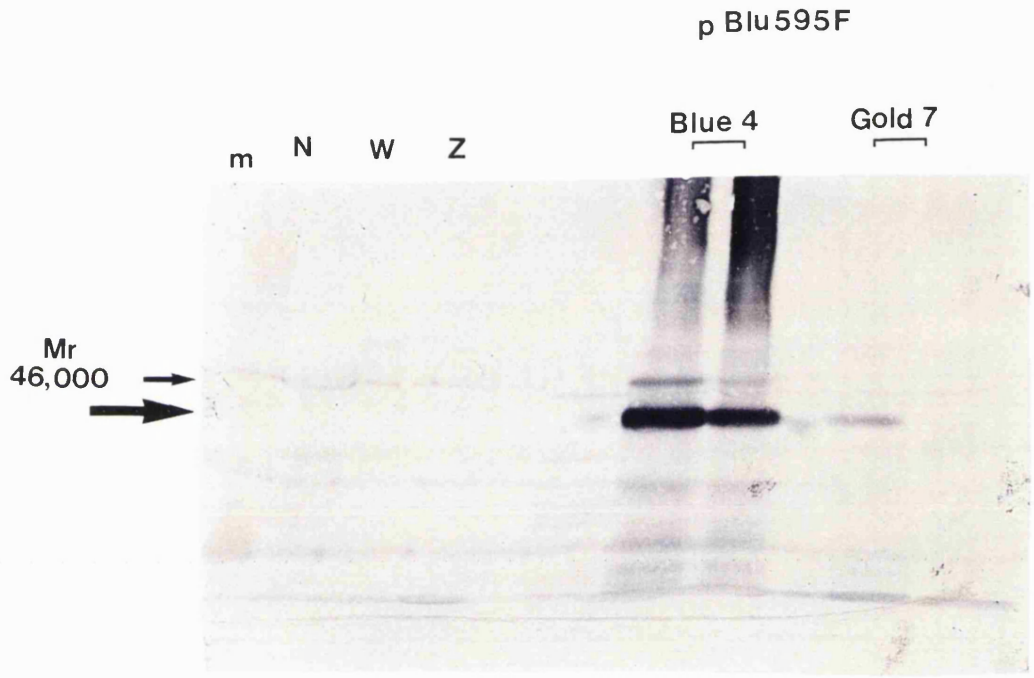

Figure 6.13: Western blot of SDS-PAGE gel, with UL97 anti-sera, for BluUL97 595F protein. The plaques Blue 4,595F and Gold 7,595F were run in duplicate alongside wildtype baculovirus DNA (W), a negative control (N) and a lacZ control (Z) all in the presence of a rainbow marker.

6.8 Discussion

The aim of chapter was to assess the ability of UL97 proteins, whose DNA sequences were derived from patient samples and were known to carry genotypic variations in UL97, to phosphorylate ganciclovir. Some considerable time was spent trying to clone the UL97 gene into pBBH using blunt ended ligation. The reasons for this difficulty were probably due to a low transformation efficiency and incomplete dephosphorylation of transfer vector DNA. Therefore, I was unable to characterise the phenotype of the mutations found, particularly in the post mortem samples, that have not as yet been previously described in the literature.

Sticky-ended ligation, however, allowed the successful insertion of both the 595F mutant and the 595L wildtype into pBBH A. The co-transfection of pBluUL97 with either Baculogold or Bac-N-Blue viral DNA appeared to be highly dependent on the quality of liposomes used and the purity of the vector DNA. The co-transfection of pBlu595F with both baculovirus DNA's was successful when performed with fresh Insectin liposomes and vector DNA prepared using large scale plasmid purification methods. DNA prepared from "mini-preparation" methods was probably not of adequate quality. The co-transfection of pBlu595L with Baculogold DNA has now been successful after many failed attempts at co-transfection. For this particular co-transfection I had re-purified the pBlu595L DNA and used a fresh stock of Invitrogen's new generation Insectin Plus liposomes and new generation Baculogold DNA. The selection of recombinant wildtype UL97 plaques is now, finally, underway.

Two recombinant plaques of pBlu595F (Blue4 and Gold7) have been grown-up into a high titre stock. Both preparations have been shown to be expressing UL97 by indirect immunofluorescence and the UL97 protein has been identified by western blotting. The UL97 protein now has to be purified and rendered soluble. The use of the BlueBacHis vector should facilitate the purification process as the six histidine residues will bind to a nickel agarose column. Other options for the purification of proteins include a Sepharose column which has recently been shown to purify a UL97 GST fusion protein to near homogeneity (He *et al* 1997). He *et al* also found that UL97 expressed in an Invitrogen BlueBac vector remained largely insoluble resulting in a phosphorylation capacity of less than one percent (He *et al* 1997). When expressed as a GST fusion protein UL97 was efficiently solubilised without loss of function. Subsequent incubation of wildtype UL97 and mutant UL97 (K355Q deletion) with [γ-^{32}P] ATP resulted in only the wildtype fusion protein being labelled (He). Further time course studies of the phosphorylation of the fusion protein led the authors to conclude that UL97 autophosphorylates at serine and threonine residues and that a single mutation at lysine 355 abolishes the incorporation of [γ-^{32}P] ATP. Mutations that alter the sequence of subdomain VI (D456 to N461 in UL97) may alter substrate binding for protein kinases and decrease but not inhibit phosphorylation. Two patients in my study were found to have previously undescribed mutations very close to this domain (449, 456 and 469) in pre-treatment samples. Neither patient became PCR negative following ganciclovir induction therapy and both patients had an early progression of retinitis. Therefore, expression of these UL97 mutations would further help to elucidate the mechanisms of ganciclovir resistance.

Unlike the study by Metzger *et al* , He *et al* his study failed to identify a natural substrate for UL97 so the substrate specificity of UL97 and its role in the resting cell remains unclear (Metzger *et al* 1994, He *et al* 1997). He *et al* were also unable to derive HCMV recombinants that were UL97 null mutants and concluded that UL97 may be essential in its entirety for HCMV replication. This may partly explain the inability of my wildtype UL97 protein to be effectively co-transfected into this baculovirus expression system. The presence of a 595 mutation, with an increased viral fitness over wildtype UL97, may have altered the expressibilty of UL97.

Future work includes the purification and solubilisation of 595 mutant and wildtype UL97 protein with a view to carrying out functional analysis with regards to monophosphorylation of ganciclovir. In the light of the problems encountered by He *et al* in the solubilisation of their baculovirus UL97 construct, several different methods for the purification and solubilisation may be required but may be facilitated by the presence of the HIS tag.

CHAPTER 7

THE USE OF CYTOMEGALOVIRUS VIRAEMIA DETECTED BY PCR TO IDENTIFY HIV POSITIVE PATIENTS AT HIGH RISK OF HCMV RETINITIS

7.1 Introduction

Despite the availability of both licensed and investigational anti-viral therapies HCMV retinitis invariably progresses, albeit not at the same rate as in untreated individuals (Holland and Shuler, 1992). Therefore, the current management of HCMV retinitis is more akin to disease limitation than eradication. At each progression, HCMV advances into normal uninfected retina reducing visual acuity with the clinical severity dependent on the site of the original lesion on the retina. It is therefore important to diagnose HCMV retinitis as early as possible in the course of the disease, when retinitis is usually still peripheral, in order to reduce the significant morbidity associated with more central lesions. On the basis of epidemiological data it has been recommended that all patients with serological evidence of previous HCMV infection and CD4 counts less than 100/μl should have a formal ophthalmological review every 3-4 months (Kuppermann *et al.* 1993; Dunn and Jabs, 1995). This approach is costly from both financial and human resource perspectives so other markers predictive of HCMV disease are needed.

The detection of HCMV DNA in the blood of HIV positive patients by PCR and antigenaemia has been shown to correlate with the current clinical diagnosis of HCMV end-organ disease in 74% and 89% of patients respectively (Hansen *et al.* 1994; Bek *et al.* 1996). However there have been no prospective studies evaluating their clinical use for the early detection of HCMV infection, prior to disease, in HIV infected individuals. This situation contrasts with the transplant setting where the detection of HCMV viraemia by molecular methods has been found to be strongly predictive of HCMV disease (Kidd *et al.* 1993; Einsele *et al.* 1995) and is used in the routine clinical management of these patients. The aim of this study was to prospectively evaluate whether HCMV viraemia, determined by PCR, can be used to target ophthalmological screening in order to diagnose HCMV retinitis at the earliest opportunity.

7.2 Methods

7.2.1 Patients

HIV positive patients attending the Ian Charleson Day Centre with previous evidence of HCMV infection (as defined by IgG seropositivity) and a CD4 count <50 cells/μl. For patients who had recently started combination anti-retroviral therapy the nadir of that individuals CD4 count was used to determine eligibility for screening.

7.2.2 HCMV PCR

DNA was extracted from 200μl of whole blood collected in a citrated tube and analysed for HCMV using qualitative PCR for gB as previously described in section 2.4. The HCMV load in qualitatively positive samples was determined using the quantitative-competitive PCR described in section 2.5.

7.2.3 Follow-up

Patients were divided into two groups based on their baseline PCR results. HCMV PCR positive patients were referred to the ophthalmology department where a full indirect fundoscopic examination through dilated pupils was performed. Patients were then followed monthly in the HIV clinic with HCMV PCR and in the ophthalmology department with indirect fundoscopy. Patients who were HCMV PCR negative were followed with 3 monthly bloods for HCMV PCR and direct fundoscopy in the clinic. Any patient in either group presenting with a history of loss of visual acuity or other symptoms such as floaters or flashing lights, irrespective of their PCR status, was seen immediately in the ophthalmology department for a full examination. Any patient with evidence of active HCMV retinitis exited from the study and was treated with standard induction and maintenance ganciclovir regimens.

7.3 Statistics

Differences in demographic details according to initial HCMV PCR status were tested for significance using the Mann-Whitney U test and the χ^2 test where appropriate. Follow-up was considered from the time of entry into the study until the time to development of HCMV disease. Patient follow-up was right-censored at the time of death or on the 31st May 1996, whichever was the earliest, if the patient had not developed HCMV disease by this date. The relationship between initial PCR status and the development of disease was examined using Kaplan-Meier methods, and was tested for significance using the log-rank test. Relationships between outcome and all baseline covariates (PCR status, age and CD4 count at entry to the study) were further studied using the Cox proportional hazards model, with each variable included as a fixed covariate (Cox and Oakes, 1984). In order to quantify the relationship between changes in HCMV viral load over time and disease development in those individuals who were initially PCR positive, Cox proportional hazards models were additionally fitted where the changing viral load was included as a time-updated covariate.

7.4 Results

7.4.1 HCMV PCR Status and the Development of HCMV Disease

During 12 months of the study, 97 patients were recruited; 27 (28%) were HCMV PCR positive at study entry and 70 (72%) were HCMV PCR negative. Demographic details of the patients are shown in Table 7.1. There were no differences in either age or CD4 count at entry to the study according to initial PCR status. However, the follow-up of the PCR positive group was significantly shorter as patients exited the study when they developed HCMV disease. 19 patients developed HCMV disease by the end of the study period, of whom 16 (84%) were initially PCR positive. There were 16 cases of HCMV retinitis (13 cases of asymptomatic peripheral retinitis in those who were PCR positive and 3 cases of symptomatic retinitis in those who were PCR negative) and 3 cases of gastrointestinal HCMV disease (all in patients initially PCR positive). Gastrointestinal disease was confirmed by both the virological and histological detection of HCMV in biopsies taken at endoscopy. All three patients with gastrointestinal HCMV disease continued to be screened monthly with HCMV PCR and by the ophthalmologist. All three patients went on to develop asymptomatic, peripheral HCMV retinitis on continued screening at a median of 5 months after starting ganciclovir induction therapy. One patient developed HCMV retinitis whilst taking oral ganciclovir maintenance therapy. The other two patients were not receiving secondary prophylaxis.

A positive PCR result at entry to the study was significantly associated with the development of HCMV disease over the one year period (p=0.0001, log-rank test, Figure 7.1). By the end of one year, 59.3% of those who were initially PCR positive had developed disease compared to 4.3% of those who were initially PCR negative. Individuals who were initially PCR positive were over 20 times more likely to develop disease than those initially PCR negative (relative hazard 20.15; Table 7.2). There was no association between the development of disease and either the CD4 count or age of the patient at entry to the study.

Table 7.1 : Details of 97 patients included in study according to initial PCR status.

Parameter		**PCR positive**	**PCR negative**	***p*-value**
Number of patients		27	70	
Age (years) :	Median	36	34	0.43
	Range	(26 - 48)	(26 - 57)	
CD4 count : (cells/μl)	Median	10	10	0.60
	Range	(0 - 70)	(0 - 100)	
Follow-up (months)	Median	4	6	0.002
	Range	(0.25 - 12)	(2 - 12)	
No. of patients (%) developing CMV disease		16 (59.3)	3 (4.3)	<0.0001

Table 7.2 : Relative hazards of developing HCMV disease according to baseline factors or HCMV load on follow-up.

	Relative hazard	95% confidence interval	*p*-value
Baseline			
CMV PCR positive	20.15	5.80 to 69.98	0.0001
Age (per 5 year increase)	0.91	0.64 to 1.31	0.62
CD4 count (per 10 cells/μl drop)	0.90	0.69 to 1.18	0.44
Follow-up			
Increase in HCMV load (per 0.25 $\log_{10}$ increase)	1.37	1.15 to 1.63	0.0004

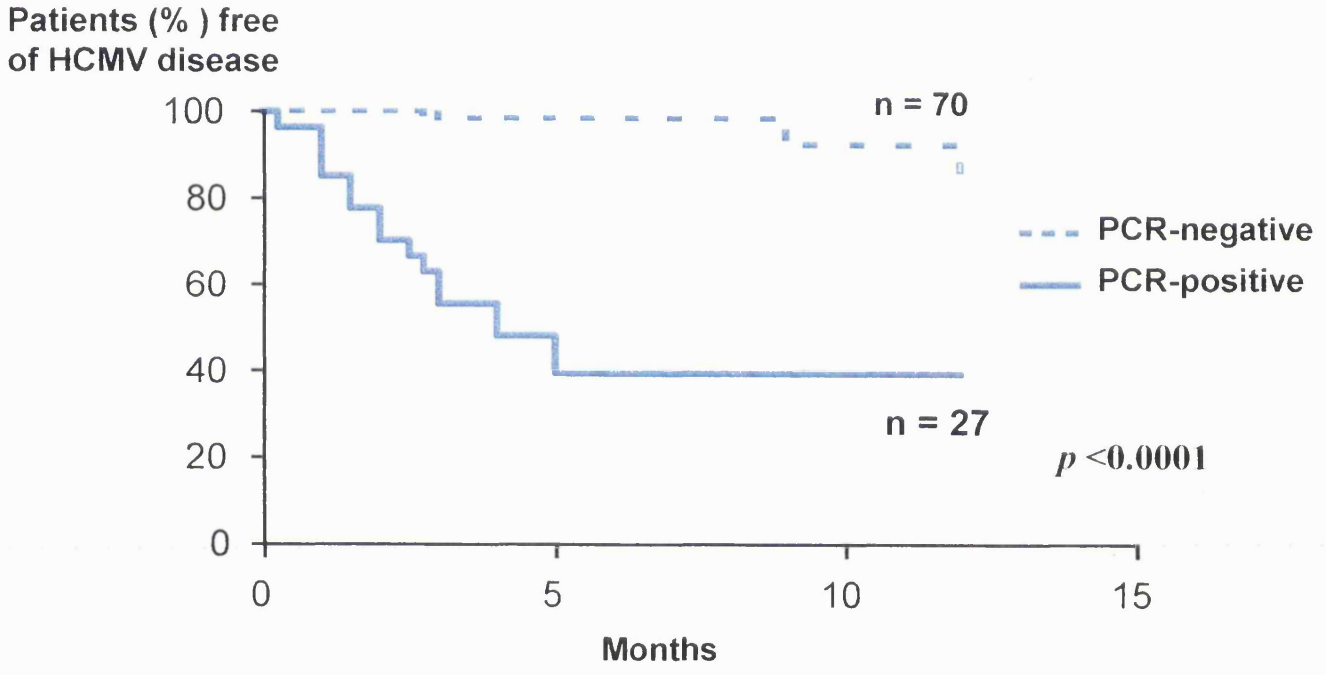

Figure 7.1: Kaplan-Meier survival analysis of the time to HCMV disease according to initial HCMV PCR status. The difference between the two groups was significant by the log rank test (p = <0.0001).

7.4.2 HCMV Load and the Development of HCMV Disease

Quantitative analysis of HCMV load in patients who were initially PCR positive showed that viral load in blood at entry to the study ranged from 3.64 to 5.66 $\log_{10}$ copies/ml (median 4.55). The median viral load at entry into the study was significantly higher in those patients who went on to develop disease (4.77 vs 4.01 $\log_{10}$ copies/ml, p=0.02). During follow-up, HCMV viral load increased in the majority of individuals (Figure 7.2) so that at presentation of HCMV disease, viral load ranged from 4.51 to 7.05 $\log_{10}$ copies/ml (median 5.28; p = 0.01 compared to baseline). In those patients who were initially PCR positive, each 0.25 $\log_{10}$ increase in viral load over time was significantly associated with a 37% increase in the likelihood of developing HCMV disease (relative hazard 1.37; 95% CI: 1.15 to 1.63, p=0.0004; Table 7.2). This effect was unchanged after adjusting for the age and CD4 count of the patient.

The three patients with gastrointestinal HCMV disease initially became PCR negative after three weeks of high dose iv ganciclovir therapy. All three then became PCR positive again on follow-up with HCMV loads that increased until HCMV retinitis was diagnosed. One patient developed HCMV retinitis despite oral ganciclovir as secondary prophylaxis. The relationship between HCMV load, HCMV disease and treatment in each of these three patients is shown in Figure 7.3.

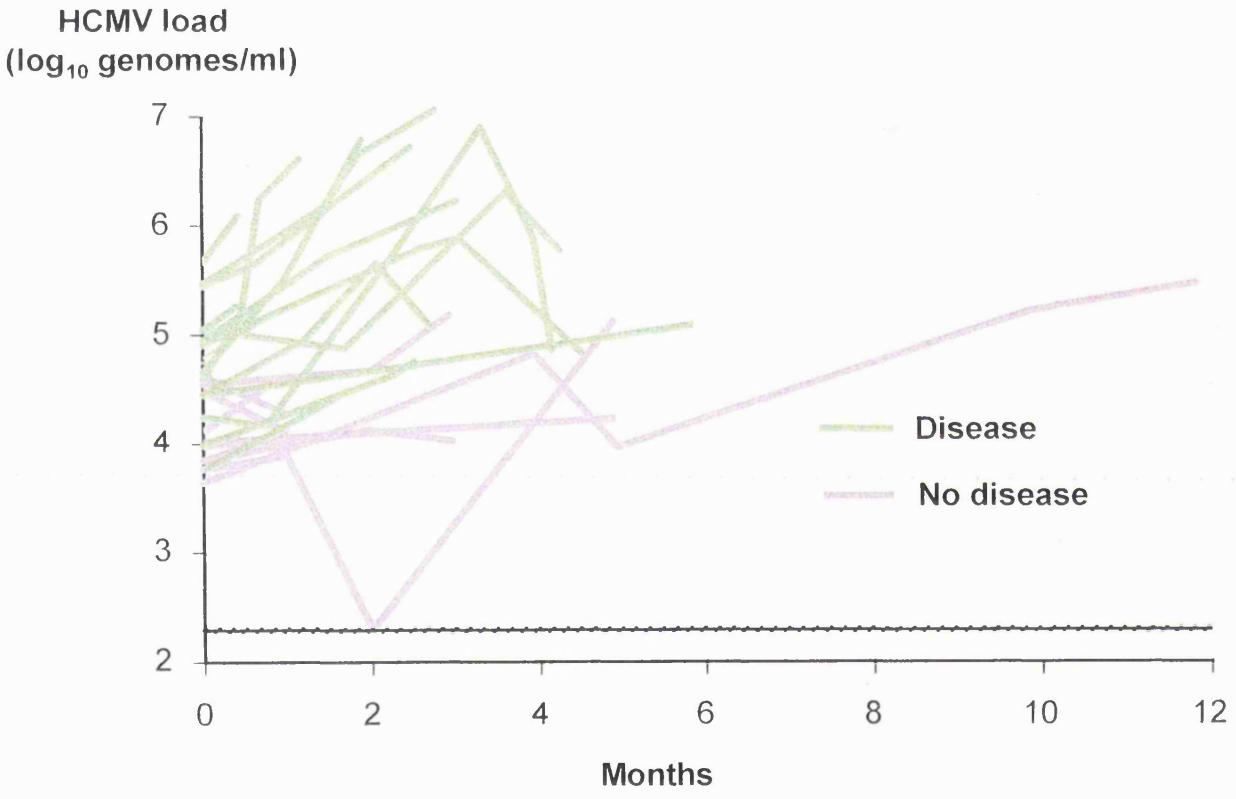

Figure 7.2: Changes in HCMV load in all PCR positive patients according to the development of HCMV disease on follow-up.

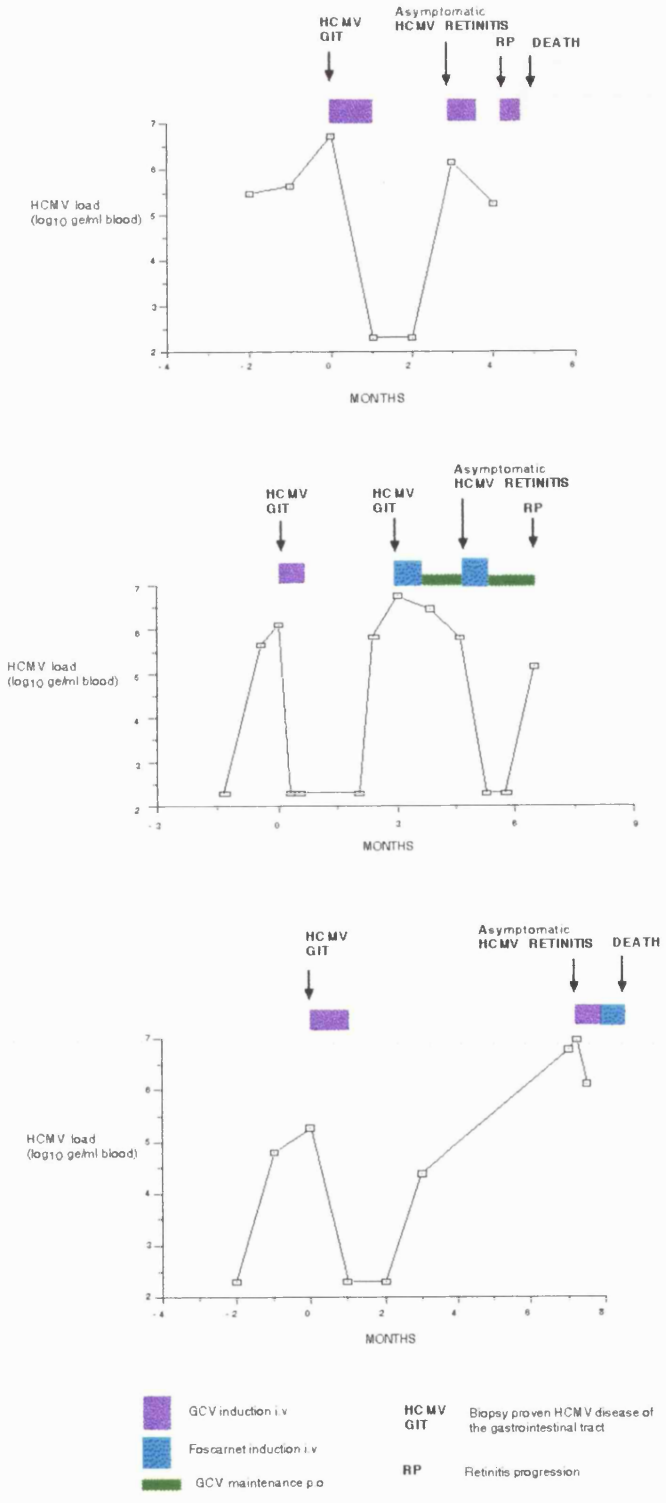

Figure 7.3: Correlation between HCMV load, HCMV disease and anti-viral therapy in three patients with HCMV GI disease who went on to develop asymptomatic HCMV retinitis on continued monthly ophthalmological follow-up.

In those patients who were initially HCMV PCR negative, there was no evidence of HCMV PCR viraemia during the study period (235 samples analysed from 70 patients) in all but the three patients who developed disease. In each of these three patients, the sample at presentation of retinitis was PCR positive but all earlier samples from these patients were PCR negative. One patient presented with retinitis four months after his last negative HCMV PCR result, having missed his three monthly appointment for his PCR. The other two patients were being followed-up regularly. One presented with retinitis just before his three monthly PCR was due, whilst the other had a negative HCMV PCR two months prior to his presentation with retinitis.

7.5 Discussion

Cytomegalovirus retinitis and gastrointestinal diseases are increasing causes of morbidity in patients with advanced HIV infection. It is generally accepted that, even with treatment, HCMV retinitis will progress in virtually all cases if patients live long enough. It is therefore important to identify retinitis whilst it is still peripheral and asymptomatic and treat promptly with the aim of maintaining central vision as long as possible. These results show that the presence of HCMV DNA in the blood predicts the development of HCMV disease. The results imply that this approach can be effectively used to direct ophthalmological services to those patients at highest risk of HCMV retinitis and to recruit PCR positive patients into trials of pre-emptive therapy.

The positive predictive value of HCMV PCR positivity and subsequent disease was 0.60 after 12 months of this study with a median follow-up for all patients to date of 5 months. This prognostic value is higher than found in previous studies where HCMV was cultured from the blood of HIV infected patients (0.35 and 0.50, (Zurlo *et al.* 1993; Salmon *et al.* 1990)), but similar to that seen with the application of HCMV PCR to transplant patients (Table 7.3). Importantly, the absence of PCR viraemia identifies patients with commensurately low risks for HCMV disease (negative predictive value 0.95).

Table 7.3: Comparison between prognostic value of HCMV PCR in HIV positive patients and renal transplant recipients.

Patient group	Sensitivity	Specificity	PPV	NPV	Relative hazard
HIV-positive	84%	86%	0.60	0.95	20.15
Renal transplant	80%	86%	0.62	0.94	12.2

In the PCR negative group three patients went on to present with symptomatic retinitis at which time all three were HCMV PCR positive. This suggests that increasing the frequency of HCMV PCR's, perhaps to a monthly basis in HCMV negative patients may increase sensitivity. An informal cost-benefit analysis of using HCMV PCR to refer patients to the ophthalmologist has been calculated. Assuming each visit to the ophthalmologist costs £50 and each qualitative PCR costs £20, the total cost of screening 70 patients in the ophthalmology department every three months for one year would cost £14,000 compared to £5,600 if those patients were to be screened using HCMV PCR. This provides not only a financial cost saving but also a saving on human resources by allowing more ophthalmology clinic time to be allocated for patients with symptoms.

The finding that intensive screening of high risk patients can pick up peripheral, asymptomatic HCMV retinitis also has important clinical implications. Peripheral retinitis progresses quickly in the absence of therapy and immediate treatment with high dose induction therapy followed by maintenance therapy is indicated (Spector *et al.* 1993). Oral ganciclovir has been shown to be adequate maintenance therapy in patients with peripheral HCMV retinitis, but is not recommended for patients with central, sight threatening retinitis who require intravenous maintenance therapy (Drew *et al.* 1995). One important advantage of this early diagnosis of peripheral retinitis is the improvement in the patients quality of life associated with the use of oral ganciclovir by obviating the need for permanent indwelling central catheters.

Since some of the patients who developed disease in the early phases of study did not have a previous negative result within the previous 6 months, it is difficult to calculate the true length of time from HCMV PCR positivity to HCMV disease. This problem has also been

encountered in two placebo controlled trials assessing the impact of oral anti-viral agents on the incidence of HCMV disease. The ACTG 204 study found that the mean time to HCMV disease in the PCR positive group receiving placebo was 11 months (Feinberg J. 1996). Further studies of patients with advanced HIV infection with regards to HCMV PCR positivity should help to determine the true time course from systemic HCMV reactivation to end-organ HCMV disease.

HCMV PCR positivity can be used in patients with advanced HIV infection to predict those patients who are likely to develop HCMV disease. The identification of such high risk patients will allow controlled trials to be designed to assess pre-emptive therapeutic strategies for the prevention of HCMV disease in the HIV infected patient population. Moreover, HCMV PCR negative patients have a very low risk for developing retinitis and need not be unnecessarily subjected to potentially toxic compounds such as oral ganciclovir. This would also allow a more cost effective approach for the use of drugs for pre-emptive therapy against HCMV disease.

CHAPTER 8

GENERAL DISCUSSION

This thesis has traced the natural history of human cytomegalovirus infection in HIV infected individuals from its reactivation in late stage HIV infection to the development of HCMV disease and an AIDS diagnosis. It has also tried to elucidate some of the factors that define an individuals disease course and influence both host and viral outcome. Many studies have looked at each of the above stages in isolation and, whilst increasing our understanding of specific areas of HCMV pathogenesis, have not necessarily looked at this process as a continuum. The aim of this thesis was, by understanding the natural history and pathogenesis of HCMV in a clinical context, to optimise the management of HCMV disease and therefore, turn away from empirical strategies of anti-HCMV therapy to structured, individualised ones.

Patients with advanced HIV infection, particularly when their CD4 counts fall below 50 cells/μl, are at risk of developing HCMV disease (Gallant *et al.* 1992; Pertel *et al.* 1992). This thesis has shown that the detection, by PCR, of HCMV in the blood of patients with advanced HIV infection confers a significantly greater relative hazard for the development of HCMV disease than by monitoring patients by CD4 count alone (relative hazards 20.15 and 0.90 respectively). This suggests that patients in such an advanced stage of HIV infection should be regularly monitored for evidence of HCMV replication in the blood. In this setting both negative and positive HCMV PCR results have important implications. Patients who are PCR negative have a low risk for developing HCMV disease. Over a 12 month study period only 3 out of 70 PCR negative patients developed HCMV retinitis and all three patients were HCMV PCR positive at presentation with retinitis. Patients in this very low risk group could be monitored with regular HCMV PCR assays rather than regular ophthalmology follow-up, therefore apportioning financial and human resources to those patients most at risk of developing disease. A patient with a positive HCMV PCR result should, in the first instance,

be followed more closely for the development of HCMV retinitis. Secondly, the high risk patient is an ideal candidate to be entered into trials of pre-emptive anti-HCMV therapy. Similar pre-emptive therapeutic regimens have been successfully employed in the transplant setting for several years (Goodrich *et al.* 1993; Schmidt *et al.* 1991). Patients in many centres are regularly monitored for evidence of systemic replication of HCMV and the detection of HCMV infection by PCR (either in PBMCs or plasma) or antigenaemia assays has been shown to correlate with the development of HCMV disease in the post-transplant period (Einsele *et al.* 1995; Kidd *et al.* 1993; van-der-Bij *et al.* 1988; Schmidt *et al.* 1996; Barber *et al.* 1996). The same information in the HIV infected individual has been more difficult to assess as there have been few prospective studies evaluating the predictive value of the detection of HCMV in asymptomatic individuals. Both HCMV PCR and antigenaemia assays have been shown to correlate with either a current clinical diagnosis of disease or a retrospective diagnosis of HCMV disease (Drouet *et al.* 1993; Bek *et al.* 1996; Salzberger *et al.* 1996). Hansen *et al* reported that their nested HCMV PCR had a relative hazard for HCMV disease of 20.3 and a positive predictive value of 0.77 (Hansen *et al.* 1994). However, this cross-sectional study included patients who had already developed retinitis. The results from the truly prospective study described in chapter 7 show that HCMV PCR positivity in blood is predictive of HCMV disease.

Although oral ganciclovir was effective in reducing the incidence of HCMV disease in patients with CD4 counts <100 cells/μl in the 1654 study (Spector *et al.* 1996), the cost of the drug (especially given its 4% bioavailability) makes such a prescribing policy not only financially prohibitive (Nicklin, 1996) but also prohibitive to the quality of life of those patients with a low risk of developing HCMV disease. Targeting the high risk population

with such therapy is financially more viable but preliminary data has indicated that oral ganciclovir is less effective in patients who were already PCR positive (Spector *et al.* 1996). Valaciclovir has also been shown to be effective at reducing the incidence of HCMV disease but the ACTG 204 study was closed prematurely due to poor tolerance of the high dose used (Feinberg *et al* 1996). Interestingly, in a subset of patients recruited to a virology study, valaciclovir was more effective in reducing HCMV disease in patients who were HCMV PCR positive in blood at baseline (ie receiving pre-emptive therapy) than in those patients who were HCMV PCR negative at baseline (ie receiving valaciclovir prophylaxis) (Feinberg *et al* 1996). In comparison to the 1654 trial of ganciclovir, it could be concluded that the bioavailabilty of ganciclovir and valaciclovir is more important than their inherent potency in individuals with systemic HCMV infection. There are several new agents (including lobucavir and cidofovir) in phase II development that are ready to move into phase III studies for the prevention of HCMV disease. One such compound is the valine ester pro-drug of ganciclovir that has a much greater bioavailability than oral ganciclovir and has the potential advantage of being taken on a once daily basis only. Based on the results from this thesis a large international trial of this compound in patients with asymptomatic HCMV viraemia is due to start in 1997.

In this thesis I have shown that HCMV load increases before the diagnosis of HCMV disease and every log increase in HCMV load increased the patients risk for HCMV disease. Due to the small numbers of patients it is not possible to identify a "threshold" value above which all patients should be aggressively treated. However, if left untreated HCMV load continues to increase, HCMV retinitis ensues and rapidly progresses and other manifestations of HCMV disease are seen. Even in the presence of ganciclovir, the HCMV load in blood at the

presentation of HCMV retinitis appears to be important. A patients HCMV load may determine their response to induction therapy, may influence their time to first progression of retinitis and ultimately their survival. Patients with high HCMV loads in blood were less likely to become PCR negative following induction therapy and have a shorter time to retinitis progression. Drouet *et al* also found that if patients with retinitis did not have a decrease in their PCR signal following ganciclovir induction therapy and remained PCR positive the risk of early relapse was high (Drouet *et al.* 1993). Similar results were seen in patients who were still HCMV culture positive after foscarnet induction therapy (Drusano *et al.* 1996). Subsequent to the results obtained in this thesis, a randomised clinical trial has been instigated to study the optimal length of ganciclovir induction therapy where patients who are HCMV PCR negative after 10 days of induction therapy are randomised to either 14 or 21 days ganciclovir. The identification of a group of patients with retinitis who could be managed successfully with only 14 days of intravenous therapy would have significant implications not only on the patients' quality of life but also on the cost of such treatment.

There was a trend for the patients, described in chapter 2, with high HCMV loads in blood at presentation with retinitis to have a shorter time to first progression that became more significant when adjusted for variables such as age, CD4 count and maintenance therapy received. Other important factors such as compliance and malabsorption of oral ganciclovir were not taken into consideration but may have influenced time to progression irrespective of load at presentation. The association between HCMV load and survival was however, very significant in this study. A high HCMV load may have been a surrogate marker for a more profound level of immunosuppression. However, in a subgroup of 24 patients in this cohort whose levels of p24 antigenaemia were factored into the proportional hazards model, the

association between HCMV load and survival was still significant. Further studies evaluating both HCMV and HIV RNA loads at baseline in patients entering controlled trials of anti-HCMV therapy would more accurately assess whether HCMV load was reflecting more severe immune dysregulation or was itself directly pathogenic. Results from this thesis of the analysis of HCMV load in AIDS patients at post mortem confirm previous findings that HCMV infection is widespread in these patients (Pillay *et al.* 1993; D'Arminio Monforte *et al.* 1992). The results in chapter 2 also showed, for the first time, that much higher HCMV loads were found in AIDS patients than the negative controls. Moreover, HCMV loads were highest in the AIDS patients in tissues where HCMV had been confirmed both by histological methods and pre-morbid clinical history.

In chapter 3, I have shown that during the early stages of maintenance therapy for HCMV retinitis the majority of patients in this study were HCMV PCR negative. Indeed, unlike the patients studied by Drouet *et al* in 1993, most patients were also PCR negative at the first progression of their retinitis and their time to retinitis progression was shorter than for PCR positive patients (Drouet *et al.* 1993). The latter group of patients not only all had a progression of their retinitis but they were also at risk of developing other HCMV related diseases. These results suggest that retinitis progression may either be due to local reactivation of HCMV or, in a smaller number of patients, reinfection from systemic viraemia that may also infect other organs. Further studies aimed at identifying the different strains involved in each new progression or the development of other diseases would help to identify the source of virus as in other immunosuppressed patient groups (Chou, 1990; Grundy *et al.* 1988).

Although HCMV PCR would not be as effective in AIDS patients to monitor response to maintenance therapy and predict progressive HCMV disease as it is in the transplant setting (Einsele *et al.* 1995; Einsele *et al.* 1991), PCR could be used to optimise an individuals maintenance therapy. The negative predictive value for HCMV PCR and retinitis progression was very low but the negative predictive value for non-retinal HCMV disease was 0.96. Therefore, whilst regular ophthalmological follow-up is still the most sensitive method for detecting retinitis progression, PCR monitoring could be used to determine which patients could be managed with topical therapy alone (such as intra-vitreal injections or intra-ocular implants) and which patients are most at risk of other HCMV disease and require systemic anti-viral therapy. This would have significant cost benefits as the combined prescription of an intra-ocular device and oral ganciclovir is likely to be prohibitive.

Following an episode of retinitis progression very few patients who were PCR positive became PCR negative after intravenous re-induction therapy. In most cases this was due to the presence of drug resistant virus. However, in cases where virus was known to be wildtype at the UL97 locus, failure to respond to therapy may have been due to the continuing deterioration of the patients immune system such that at each retinitis progression there is insufficient host cellular immunity to help eradicate viraemia. A second explanation, that anti-viral therapy is concentrated in specific cellular sites and does not have an impact on viraemia, is more speculative but may be applicable to cidofovir. In the five patients in this study who received cidofovir, two remained PCR positive with little or no reduction of HCMV load following the initiation of cidofovir therapy, and the other three became PCR positive. Despite the persistently high levels of HCMV viraemia in these five patients, all five had immediate stabilisation of their retinitis with no further episodes of progression.

However, two of these patients developed hypoadrenalism and one patient died from acute pancreatitis that was probably HCMV related. It appears therefore, that cidofovir may be concentrated in the retina to good effect but with little benefit on virus in blood. Further studies looking at the presence of mutations in UL54 of these 5 patients will help to determine whether cidofovir resistant virus was a factor in this persistent DNAemia.

The results presented in chapter 4 showed that the majority of patients who had an episode of retinitis progression did so in the absence of viraemia. This suggests that the presence of HCMV strains in the blood with reduced susceptibility to ganciclovir was not the cause of most cases of first retinitis progression. This does not, however, exclude the possibility of the reactivation of resistant strains in vitreous fluid as the aetiology for progression. In the patients who became PCR positive at some time on maintenance therapy (n=21), 13 had mutations in UL97 demonstrated in blood (or CSF in one case) either by DNA sequencing (n=4) or by PMA (n=9). Most of the coding mutations found by conventional sequencing methods in this study have not been described elsewhere (Chou *et al.* 1995; Wolf *et al.* 1995; Hanson *et al.* 1996) and some were associated with a reduced response to induction therapy and retinitis progression. As these patients did not have resistant isolates grown in cell culture phenotypic studies were not performed on these mutant strains since there were multiple coding mutations in each patient further studies such as site directed mutagenesis would be required to identify if these mutations were capable of conferring ganciclovir resistance to Ad169.

There was some correlation between the presence of mutations at more than one of the codons screened by PMA with aggressive bilateral disease recalcitrant to treatment. However, there were exceptions to this scenario as two individuals who exhibited an identical pattern of rapid

disease progression both remained PCR negative after induction therapy and throughout maintenance therapy. The development of a rapid assay, such as the PMA, that can determine the UL97 sequence at the codons most frequently associated with ganciclovir resistance (Chou *et al.* 1995) is likely to be useful in the clinical setting. In several cases, the results obtained in this thesis were used to optimise a patients anti-viral therapy. In the majority of patients however, who remain PCR negative, clinical management will still be centred around achieving adequate levels of anti-viral therapy in the desired tissue compartment to prevent disease progression.

In order to study the phenotypic nature of some of the new mutations described in this thesis I attempted to clone various UL97 mutations into a baculovirus expression system. I have successfully cloned and expressed a F595 truncated mutant of UL97 in a recombinant baculovirus system and produce recombinant protein. To date, UL97 has been expressed in heterologous systems and has been shown to induce ganciclovir phosphorylation (Metzger *et al.* 1994; Michel *et al.* 1996) as well as autophosphorylate itself at three sites in subdomains VI and VIII (He *et al.* 1997). Mutations mapping to the 460 region may disrupt substrate recognition whereas mutations in or around region VIII, that contains codon 520, may interfere with the function of F521, an amino acid conserved amongst serine/threonine protein kinases. These mutations in UL97 may decrease the phosphorylation of ganciclovir without affecting the phosphorylation of its natural substrate (as yet unknown) and so suggest that UL97 is essential for HCMV replication. Therefore, to express wildtype UL97 in a baculovirus expression system the whole of the UL97 ORF, including the lysine at codon 355 (He *et al.* 1997), may be necessary for the effective production of wildtype protein.

Purification and solubility of recombinant UL97 was found to be a problem by He *et al* who were unable to detect any enzymatic activity associated with a UL97 fusion protein expressed in *E.coli* (He *et al.* 1997). Expression of UL97 in a baculovirus system resulted in a protein with little enzymatic activity that was largely insoluble (He *et al.* 1997). The whole of the UL97 ORF was then successfully expressed and purified as a GST fusion protein using recombinant baculovirus. The protein was purified to near homogeneity using SP Sepharose chromatography. The mutant UL97 protein expressed in this thesis had been cloned into a baculovirus vector containing six histidine residues proximal to the multiple cloning site to aid protein purification using nickel agarose columns. Future work includes the purification and solubilisation of this F595 mutant protein to assess its enzymatic activity as well as expression of a His tagged wildtype truncated UL97 for comparative enzymatic analysis.

In an attempt to gain a better understanding of the *in vivo* dynamics of HCMV results generated from the PMA in this thesis were used to study the viral fitness of different UL97 mutations in the presence of anti-viral therapy and estimate a generation time for HCMV *in vivo*. Both F595 and I460 mutations were approximately 3-6% more fit than their wildtype counterparts in the presence of ganciclovir. This is a much larger fitness gain than seen for a single TCC mutant at codon 215 of the HIV RT with a fitness gain over mutant between 0.4 to 2.3% (Goudsmit *et al.* 1996). F595 remained mutant in the presence of foscarnet (probably due to a double mutation in UL54 conferring cross resistance to foscarnet as previously described, (Baldanti *et al.* 1996)) but S595 and I460 reverted to wildtype in the presence of cidofovir. S595 had a fitness loss in comparison to wildtype of 3.6% whilst I460 had a much greater fitness loss of over 12%. However, these two mutations were found in the same patient and may confound each other and further studies including DNA sequencing would

be needed to truly estimate the proportion of these virus strains under different anti-viral pressures.

The generation time for HCMV, calculated from the results in this thesis, was found to be approximately one day. This is much faster than the accepted *in vitro* propagation rate for HCMV and suggests that HCMV replication *in vivo* is a much more highly dynamic process than previously thought comprising predominately of virus produced from newly infected cells. It is this highly active process that is inhibited first by anti-viral therapy and can be seen in the rapid half life of HCMV load reduction in the bi-phasic response to ganciclovir induction therapy. Such bi-phasic models have been suggested for the *in vivo* dynamics of HIV infection where productively infected cells have been estimated to have a half-life of 1.6 days and plasma virions a half-life of 0.24 days (Perelson *et al.* 1996). Perelson *et al* concluded that 2-3 weeks of potent anti-retroviral therapy could "eradicate" HIV infection in these two compartments. However, Ho suggested that it may up to three years of such therapy to abolish HIV infection in a third sequestered compartment containing long-lived cell populations (David Ho, XI International Conference on AIDS, Vancouver 1996). Further *in vivo* studies on the dynamics of HCMV infection may also help to define treatment strategies to suppress HCMV into a latent state.

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