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Investigation of Herpes simplex virus and Cytomegalovirus Infection in the Immunocompromised Host

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for the degree of

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by

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Abstract

The remit of this thesis was to examine the role of HSV and CMV infections in patients with AIDS and in bone marrow transplant recipients. PCR was used to monitor the efficacy of a novel drug, valaciclovir, in AIDS patients at risk of CMV disease. Valaciclovir was found to be effective at reducing the excretion of CMV in patients who were baseline PCR-positive, and in patients with advanced HIV disease, the drug delayed the onset of CMV end-organ disease.

A confirmatory assay based on an oligonucleotide probe conjugated to alkaline phosphatase was used to evaluate a number of samples from the ACTG 204 study that produced non-specific results using PCR.

Quantitative PCR was used to retrospectively monitor fluctuations in viral load in the blood of BMT recipients. When the relative contribution to CMV disease of risk factors such as recipient CMV serostatus, virus load, T-cell depletion, and acute-GvHD was assessed, virus load was found to be the most significant risk factor for disease. In univariate statistical analysis, recipient seropositivity was also identified as a significant risk factor for CMV disease. Immune modulation and the dynamics of virus clearance from the blood were assessed in order investigate the efficacy of antiviral intervention with ganciclovir.

Finally, nine herpes simplex virus isolates from 3 HIV patients and one BMT patient, which were resistant to ACV in vitro, were subjected to phenotypic and genotypic analyses. All the viruses were found to be TK deficient, and genotypic analysis showed the presence of a number of mutations including single base deletions and coding substitutions.

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Abbreviations

32P	Phosphate (radioisotope)
^{35}S	Sulphur (radioisotope)
aa	amino acid
A-GvHD	Acute Graft verses Host Disease
ACV	Aciclovir
AIDS	Acquired Immune Deficiency Syndrome
ALL	Acute lymphocytic leukaemia
ATG	Anti-thymocyte globulin
ATP	adenosine triphosphate
BAL	Bronchoalveolar lavage
bid	Bis die (twice a day)
BMT	Bone Marrow Transplant(ation)
CID	cytomegalic inclusion disease
CMI	cell mediated immunity
CML	Chronic Myelogenous Leukaemia
CMV	Cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CSF	Cerebrospinal fluid
CTL	cytotoxic T lymphocytes
CxR	Chest X-ray
DEAFF	Detection of Early Antigen Fluorescent Foci
DNA	deoxyribonucleic acid
DNA pol	DNA polymerase
EBV	Epstein-Barr Virus
EDTA	ethylenediaminetetraacetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FITC	fluoroscein isothiocyanate
FOS	Foscarnet
G-CSF	Granulocyte- colony stimulationg factor
GCV	Ganciclovir
GM-CSF	Granulocyte-macrophage colony stimulating factor
GvHD	graft verses host disease
HLA	Human Leucocyte Antigen
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HSV	Herpes simplex virus
I/V	Intravenous
IE	immediate early
Ig	immunoglobulin
IR	inverted repeat
ISH	in situ hybridisation
Kb	kilobase
KSHV	Kaposi's Sarcoma herpes virus

LAT	latency associated transcript
MHC	major histocompatibility complex
MIEP	major immediate early protein
mRNA	messenger ribonucleic acid
MRD	minimal residual disease
NIEP	non-infectious enveloped particle
NK	natural killer
NPV	negative predictive value
ORF	open reading frame
PCR	polymerase chain reaction
pi	post infection
PFA	Foscarnet
PML	peripheral mononuclear lymphocytes
PMNL	polymorphonuclear leucocytes
PPV	positive predictive value
qds	quater die sumenus (four times a day)
QPCR	Qualitative PCR
QCPCR	Quantitative PCR
RE	restriction enzyme
RH	Relative Hazard
RR	Relative Risk
RT-PCR	reverse transcriptase PCR
STD	sexually transmitted disease
T ALL	T-cell acute lymphocytic leukaemia
TAP	transporter associated with antigen processing
tid/tds	ter die sumendus (three times a day)
TK	thymidine kinase
TR	terminal repeat
UL	unique long
US	unique short
VACV	Valaciclovir
vhs	virion host shutoff
VZV	Varicella zoster virus
WBC	white blood cells

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Chapter 1

General Introduction

1.1 Classification of Herpesviruses

Membership of the family Herpesviridae is based on the structure of the virion. A typical virion consists of a core containing a linear double stranded DNA, an icosahedral capsid around 100 - 110 nm in diameter, composed of 162 capsomeres, a tegument and an envelope. The envelope forms the outer cover of the virion and has a number of protrusions or spikes made up of virally encoded glycoproteins. The overall diameter of the virions varies from 120 -300 nm.

Herpesviruses are divided into three subfamilies based on biological properties.

1.1.1 The Alphaherpesvirinae

The viruses in this subfamily have a variable host range, a short replication cycle and spread rapidly in tissue culture. They have a lytic effect on infected cells and have a tropism for sensory ganglia cells, establishing latency in these cells. Members of this subfamily are the Herpes simplex viruses types 1 and 2, (HSV 1& 2, or human herpesviruses 1 and 2), and Varicella zoster virus, (VZV, or human herpesvirus 3 HHV3).

1.1.2 The Betaherpesvirinae

Betaherpesviruses are more restricted in their host range and have a longer replication cycle. Infection in cell culture progresses slowly (around 21 days for cytopathic effect to be apparent) and latency can be established in a variety of host cells. Human cytomegalovirus, (HCMV or human herpesvirus 5, HHV5), murine CMV (MCMV), and HHV6 and 7 are members of this subfamily. For the purposes of this thesis, HCMV will be referred to as CMV.

1.1.3 The Gammaherpesvirinae

All members of this subfamily replicate in lymphoblastoid cells, and the viruses are specific for either T or B cells. Lytic infection can be seen in some epitheliod or fibroblastic cells, and latent infection is often seen in lymphoid tissue. Epstein Barr virus (EBV), and the newly discovered Kaposi's sarcoma virus, KSHV or HHV8 belong to this subfamily.

1.2 Herpes simplex viruses 1 and 2

Herpes simplex viruses were the first herpesviruses to be discovered and have been extensively studied since then. Schenweiss *et al.,* (1962), demonstrated that there were two specific serotypes, HSV 1 and 2, which are now designated human herpesvirus 1 and 2 under the rules of the International Conference for Taxonomy of viruses (Roizman *et al.,* 1981).

1.2.1 Virion Structure

The HSV virion is made up of four elements:

a) an electron dense core

b) an icosadeltahedral capsid which surrounds the core,

c) a tegument around the capsid, and

d) an outer envelope with glycoprotein spikes at its surface.

There are around 40 virion polypeptides that have been identified, of which eleven are glycosylated. These are gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM. Glycoprotein B is required for infectivity, gD is also related to viral infectivity and is the most potent inducer of neutralising antibodies. Glycoprotein C binds to the C3b component of complement, and gE binds to the Fc portion of IgG. Glycoprotein G provides the antigenic specificity for HSV. The role of the other glycoproteins is not well elucidated as yet.

The envelope of the virus contains at least two, and possibly four non-glycosylated membrane proteins, and Stannard *et al.,* (1987), have shown that the spikes on the envelope are virally encoded glycoproteins.

Three types of viral capsid have been reported:

A capsid are empty capsids

B capsids are capsids with scaffolding for DNA but without DNA, and

C capsids which contain DNA.

1.2.2 Viral DNA

The double stranded linear HSV DNA is packed in the form of a toroid, with the ends held together in such close proximity to each other that the DNA appears to be circular. The HSV genome is approximately 150 kbp in length, with a G-C content of 68 and 69% for HSV 1 and 2 respectively. Two covalently linked components, L (long), and S (short) both consist of unique sequences flanked by inverted repeats.

The L and S components can invert relative to each other and yield linear isomers: P_I (prototype), I_L (inversion of the L component), I_S (inversion of the S component) and I_{SL} (inversion of both components). The internal repeat sequences are not essential for the growth of virus in cell culture.

1.2.3 HSV Polymorphisms

1.2.3.1 Intertypic Variation

HSV 1 and 2 differ in their restriction endonuclease (RE) cleavage sites and in the sizes of their viral proteins. The two types are distinct enough to be characterised serologically with type specific monoclonal antibodies.

1.2.3.2 Intratypic Variation

Differences between HSV1 strains at the DNA level result from base substitutions, which may add or eliminate a restriction site, or change an amino acid, or vary the number of repeat sequences present in regions of the genome.

The restriction endonuclease (RE) cleavage patterns tend to remain the same in isolates from the same patient over a number of years, but variability of the repeat sequences is unstable. Polymorphism of RE sites is frequently noted in unrelated individuals, thus allowing tracing of transmitted infection from patient to patient. Sakoaka *et al.,* (1994), reported a clustering of polymorphisms based on race and geographical location, and suggested a possible host involvement in the evolution of HSV1. What is perhaps more likely is that the virus has co-evolved with its host and a conservation of these co-evolved mutations is seen in geographically distinct populations.

1.2.3 Replication of HSV

The major events in the life cycle of HSV can be summarised as follows:

a) virus attaches to the cell surface,

b) fusion of the viral envelope with the plasma membrane,

c) transport of de-enveloped capsid to nuclear pores and release of DNA into the nucleus.

d) transcription, replication of viral DNA, assembly of new capsids in the nucleus.

Viral gene expression is ordered in a sequential cascade. Many of the gene products are enzymes and DNA binding proteins involved in DNA replication. Much of the viral DNA is synthesised by a rolling circle mechanism, which produces concatemers which are cleaved into monomers before being packaged into capsids.

Assembly is in stages: DNA is packaged into pre-assembled capsids, the virus matures and acquires infectivity by budding through the inner lamellae of the nucleus. This process normally takes 18 - 24 hours in permissive cell culture.

1.2.4 Viral Genes

Transcription of viral DNA takes place in the nucleus and all viral proteins are synthesised in the cytoplasm (Honess and Roizman, 1974, Preston, 1979, Sandri-Goldin & Mendosa, 1992).

Alpha genes are expressed first: There are five immediate early or α proteins ICP0, ICP4, ICP22, ICP27 and ICP47. The immediate early genes have regulatory and immunomodulatory functions (Roizman and Sears, 1990; York *et al.,* 1994). ICP0 is a nuclear phosphoprotein, which transactivates viral promoters at the transciptional level, and this transactivating activity is also required for reactivation of the virus (Cai 1993). ICP0 mutants grow inefficiently and are reactivation impaired. The immediate early protein ICP27 has been identified as an essential virus protein that is involved in the switch from early to late gene expression (Winkler *et al.,* 1994). Alpha polypeptide synthesis reaches its peak at around 2 - 4 hours post infection, but the polypeptides continue to accumulate throughout the replication cycle. Alpha gene products have regulatory functions and are required for the synthesis of other polypeptides.

Beta genes are expressed at very low levels in the absence of α products, and expression of β is increased rather than decreased in the presence of drugs that block DNA synthesis. Peak synthesis is at around 5 - 7 hours post-infection. Beta-1 proteins are ICP6, which forms part of the viral ribonucleotide reductase, and ICP8, which is the major DNA binding protein. Viral thymidine kinase and DNA polymerase are β2 polypeptides. The β proteins signal the onset of DNA replication.

The gamma genes consist of γ1 and 2. Gamma-1 specifies for glycoproteins B and D and γ34.5, together with the major capsid protein ICP5. These proteins are expressed relatively early in infection. Gamma-2 proteins are expressed later and are exemplified by glycoprotein C (gC), (UL44), and pUS11.

Genes that are dispensable for growth in tissue culture code for a number of different proteins which are involved in virus entry, regulation of gene expression, post-translational modification of expressed proteins, inhibitors of host immunity and infectivity. It may be that viral genes encode proteins which are the same as or interchangeable with cellular gene products, and are therefore dispensable, e.g. the thymidine kinase gene, and genes specifying for ribonucleotide reductase. Alternatively, it may be that the virus carries several different genes specific for entry and replication in a variety of different cells, (e.g. the non-essential glycoproteins), and there may be adequate functional homologues expressed by the cultured cells to allow entry and replication of certain deletion mutants not expressing some viral genes.

HSV specifies a large number of proteins involved in nucleic acid metabolism and DNA synthesis. These proteins fall into two categories: proteins that are essential for viral origin-dependent amplification of DNA, and enzymes involved in nucleic acid metabolism e.g. thymidine kinase, ribonucleotide reductase, deoxyuridine triphosphatase, uracil glycosylase and alkaline exonuclease (Liptak *et al.,* 1996). Seven genes mapping to the L component (ORFs UL5, 8, 9, 29, 30, 42, 52) specify a DNA polymerase (UL30), a single strand specific DNA binding protein designated ICP8 (UL29), a protein binding to three sites at or near the origin of viral DNA synthesis (UL9), a protein which binds to double stranded DNA and confers processivity on the DNA polymerase (UL42), and three additional proteins (UL5, UL8, and UL52) which form a complex that functions as a primase, and, in the presence of UL8, a helicase (Kuhn *et al.,* 1996). The DNA polymerase in particular, has been the subject of numerous studies because of its sensitivity to a variety of compounds such as phosphonoformate (Foscarnet), and some DNA polymerases have been found to be resistant to a variety of drugs inhibitory to wild type viruses such as Foscarnet and nucleoside analogues such as aciclovir. The impact of mutations in this gene giving rise to resistance to these compounds on antiviral therapy is discussed later in this chapter.

1.2.5 Virulence of HSV and the Host Response to Infection

Herpesvirus infected cells show a characteristic shutting-off of the host's macro molecular metabolism. Host DNA synthesis is shut off by means of the γ protein vhs (virion-host shutoff) and by ICP27. The vhs is a 58 Kda protein encoded by the UL41 gene within the tegument of the infecting virus. Shut-off can occur immediately following virus infection even though the UL41 gene itself is expressed as a late gene (Strelow and Leib, 1995). Protein synthesis declines rapidly, as does glycosylation of host proteins. Structural changes in the cell include disaggregation of the nucleus and changes in the cellular membranes. The cells typically appear ballooned and multinucleated in tissue culture.

Virulence is the ability of the virus to cause disease. Symptomatic HSV infection in humans manifests as primary or recurrent epithelial lesions, disseminated disease and encephalitis. In healthy, immunocompetent individuals encephalitis is highly pathologic but rare. Prompt treatment with intravenous (IV) aciclovir followed by long term oral treatment has greatly reduced mortality and morbidity associated with this condition (Prentice *et al.,* 1997).

Growth of virus in the central nervous tissue (CNS) is usually the means by which the neurovirulence of HSV is measured in experimental animal models. Virus injected directly into the CNS tissue of laboratory animals is used to measure the capacity of the virus to destroy enough of CNS tissue to cause death before the host immune system blocks the spread of virus. Thus the growth of virus in the CNS required to produce a certain level of mortality in 50% of inoculated animal can be quantitated and related to virus multiplication.

The natural history of HSV infection is influenced by specific and non-specific host responses. Genetic make-up, macrophages, natural killer cells, specific T-cell populations, antibodies and cytokine responses are thought to contribute to the host defence repertoire against HSV infections. Host responses to infection may influence the acquisition of disease, the severity of the infection and the subsequent reactivation

of HSV. Clearly, the host responses in the neonate and in immunocompromised hosts have to be defined separately from those of the healthy host. The pathology caused by HSV in immunocompromised hosts will be discussed later in this chapter.

1.2.7 Immune Evasion and The Role of Apoptosis in Virus Survival

Like other DNA viruses, HSV has evolved mechanisms to avoid the host immune responses in order to establish a persistent or latent infection. New virus production must occur throughout the life of the host, not to an extent that is noticed by the host, but enough to produce an inoculum capable of infecting a new host. The host must remain healthy if the virus is to be able to spread to new hosts, so there is a considerable disadvantage to causing disease. Thus, herpesviruses need to be able to a) acutely infect the host to establish a new infection with little ill effect; b) establish a latent or persistent infection maintaining an intact viral genome in the host for several decades and; c) reactivate from latency, enter the productive cycle and produce sufficient virus to infect new host cells in the face of a fully primed immune system. The virus is able to do this because of its large genome with abundant coding capacity for genes with highly specialised functions to modify the host environment. These include genes that prevent apoptosis in differentiated cells, that interact with antibody and complement, and that prevent the induction of CD8+ cytotoxic T-cells.

HSV-1 also expresses two types of IgG Fc receptors on the host cell surface, one of which, a complex of gE and gI, binds monomeric IgG and the other, consisting of gE alone, binds polymeric IgG (Dubin *et al.,* 1991). The Fc receptor is thought to have a role in protecting infected cells or virions from the host antibody response. HSV also appears to protect itself from the host's CD8+ cytotoxic T-cell response. Studies have shown that expression of the sequences containing the α47 gene in human fibroblast cells leads to resistance to lysis by CD8+ CTLs due to retention of the MHC Class I molecules in the cytoplasm and a lack of peptide presentation on the cell surface (York *et al.,* 1994; Hill and Masucci, 1998). ICP47 has also been shown to interact directly with the transporter associated with antigen processing (TAP), thus inhibiting peptide translocation into the endoplasmic reticulum (Hill *et al.,* 1995).

HSV-1 does not induce apoptosis in all cell types and the basis of selective programmed cell death is not yet understood. It may be that the viral gene products that normally block apoptosis are poorly expressed or fail to function in some cell types. HSV-1 can both induce apoptosis and inhibit it, the outcomes dependent on the target cell types and the conditions of the infection. For instance, in cells where a lytic infection is necessary in order to disseminate the virus apoptosis is induced, and in cells such as those of the neuronal ganglia in which a persistent or latent infection is to be established apoptosis is inhibited. Activated T lymphocytes play an important role in the clearance of local herpesvirus infections (Nash, 1987), and their destruction could delay virus clearance, facilitating the spread of virus in the host. However, although T cell killing is important for virus clearance, the virus has evolved to kill these cells by inducing apoptosis rather than to inhibit apoptosis and replicate in these cells. It may be that the induction of apoptosis provides a means for the virus to establish a balanced long-term relationship with the host immune system, sacrificing some virus and some lymphocytes while permitting other cells to undergo infection and thereby promoting virus survival. The mechanism by which the virus induces programmed cell death is unclear (Keiff, & Schenk, 1998). However, HSV type 1 contains a gene, $\gamma 1$ 34.5, which is not required for virus replication in culture but has been shown to prevent neuroblastoma cells_from switching off the protein synthesis which is associated with neuronal apoptosis (Chou & Roizman, 1992). Viruses with mutations in the $\gamma_1 34.5$ gene have been shown to have an associated loss of the capacity to cause disease and death, but can reactivate spontaneously (Javier *et al.,* 1988, Perng *et al.,* 1996). HSV-1 also encodes a protein serine/threonine protein kinase US3, which can block apoptosis induced by virus infection (Leopardi *et al.,* 1997). Finally, HSV-2, but not HSV-1, has been shown to inhibit the cell surface expression of the Fas ligand on infected T lymphocytes (Seig *et al.,* 1997). The interaction of the Fas ligand with the Fas receptor is one of the principal mechanisms by which cytotoxic T cells_kill target cells; the interaction with the Fas ligand leads to apoptosis in cells expressing the receptor. The ability of HSV-2 to infect activated T cells and to block Fas ligand expression would probably interfere with the ability of CTLs to abort reactivated local HSV-2 infections and may have a role in enhancing

the frequency and severity of clinically apparent recurrent HSV-2 infections. As yet, the HSV-2 gene product responsible for the downregulation of Fas ligand expression has not been identified (Keiff & Schenk , 1998).

1.2.8 Latency

HSV establishes latency in sensory neurones (Roizman and Sears, 1996). An advantage of maintaining a persistent infection in neuronal cells is that these cells are long lived and can therefore harbour virus indefinitely; thus allowing HSV to maintain its genome with little or no protein synthesis. This, combined with the lack of the lack of Class I expression on neurones, means that HSV does not need a specific immune avoidance mechanism for latency. Expression of over 75 genes of the HSV genome is suppressed in latency. A single region of the genome remains transcriptionally active during latency, producing a family of latency associated transcripts (LATs). LAT expression has become the hallmark of latent infection, detectable by in situ hybridisation (ISH) in neurones for years after primary infection. Ramakrishnan *et al.,* (1996), have used an in situ reverse transcriptase PCR (RT-PCR) to show that large numbers of neuronal cells are latently infected with HSV.

1.2.9 Laboratory Diagnosis of HSV Infection

Swabs taken from oral or recto-genital lesions can be inoculated onto monolayers of fibroblast or Vero cells. Virus replication is rapid and a typical cytopathic effect (CPE) is seen in 1 - 7 days. Monoclonal antibodies may be used to distinguish the two serotypes HSV 1 and 2, if required. HSV infection in the neonate can manifest as disseminated disease, central nervous system (CNS) infection, or disease of the skin, eye and mouth. For this group of patients, and for adults with herpes simplex encephalitis, a more sensitive diagnostic system is required.

HSV encephalitis is one of the most common encephalitides occurring in immunocompetent patients. It is usually caused by HSV-1. Serological tests of CSF and serum can give some information, but these are not usually reliable until 3 - 12

days after the onset of neurological symptoms. Currently, diagnosis relies primarily on the detection of viral DNA in CSF by PCR analysis. Aurelius *et al.,* (1991) used a nested PCR method to detect HSV in CSF samples, and detected HSV DNA in 42 of 43 patients with proven HSV encephalitis, with 41 first samples positive. The PCR negative patient was one had been treated with aciclovir for 20 hours after onset of symptoms.

In a study of 202 infants with HSV disease (Kimberlin *et al.,* 1996) PCR was used to compare vidarabine with aciclovir for the treatment of neonatal infection. HSV DNA was detected in 76% of infants with CNS disease, 93% of these with disseminated infection and 24% with skin, mouth and eye infection. Although the tests were highly sensitive (80%) and specific (71%), the authors advised caution in the application of CSF PCR in the absence of more studies. Since this work other studies have demonstrated that PCR is a highly reliable, rapid, and minimally invasive in comparison with brain biopsy, which for years had been the gold standard for the diagnosis of HSV encephalitis. Also, because of the sensitivity of the PCR analysis, a spectrum of clinical syndromes caused by infection of the nervous system due to either HSV1 or HSV 2 has been identified, including forms with an atypical course or unusual localisation (Cinque *et al.,* 1998; Cleator *et al.,* 1996).

1.2.10 Herpes Simplex Infection in the Immunocompromised Host

Patients immunocompromised either iatrogenically or by underlying disease are at risk for severe HSV infections. Renal, hepatic, bone marrow and cardiac transplant recipients are all at increased risk for HSV infection and disease post-tranplant (Pass *et al.,* 1978; Whitley *et al.,* 1984), as are patients immunocompromised by HIV infection. In organ tranplant patients the presence and level of HSV specific antibodies pre-treatment is indicative of an increased risk of recurrence. Disease in these patients may be progressive, involving the respiratory tract, oesophagus and the gastrointestinal tract. The severity and progression of disease is directly related to the degree of immunosuppression.

Reactivation of HSV is a major cause of morbidity and occasional mortality after bone marrow transplant. Eighty percent of seropositive patients who receive marrow reactivate virus after transplant. (Meyers *et al.,* 1980; Saral *et al.,* 1981; Wade *et al.,* 1984).

The absence of a specific cellular immune response to HSV in the first month post-transplant allows infection to become severe and debilitating, particularly in combination with mucosal damage from radiation and chemotherapy where mucocutaneous HSV infection can be severe.

ACV is a safe and effective treatment for HSV infections, although recurrences can be frequent if the dosage is too low, or if drug is removed for short periods. Since the work of Wade *et al.,* (1983), and Saral *et al.,* (1981), oral ACV has been routinely used for prophylaxis in the BMT patients to control HSV infections. Although viral shedding often remains, recurrences of lesions and associated morbidity is reduced. ACV resistant HSV infection in these patients can result in disease that is progressive. The clinical implications of drug resistant mutants in the BMT patient are discussed in Chapter 6 of this thesis.

1.2.11 Antiviral treatment of HSV Infection

1.2.11.1 Aciclovir

Aciclovir or 9-(2 –hydroxyethoxymethyl: Zovirax) guanine (ACV) is a nucleoside analogue which is an extremely effective inhibitor of HSV replication, with very little toxicity for uninfected cells. A diagrammatic representation of the mechanism of action of ACV in HSV infected cells is shown in Figure 1.1. The process of ACV phosphorylation in the infected cells yields the active antiviral agent, ACV triphosphate, which behaves like a normal deoxynucleoside triphosphate; thus it competes with deoxyguanosine triphosphate for the viral DNA polymerase. ACV triphosphate is a more potent inhibitor of herpesvirus DNA polymerases than of cellular DNA polymerases (Elion, 1982). ACV triphosphate inhibition of the DNA polymerases cannot be reversed by the addition of excess deoxyguanosine or more template. So ACV acts first as a substrate for, and then as an inhibitor of viral DNA polymerase. The successful competition for the polymerase gives rise to a

triphosphate/DNA pol/template complex which results in the incorporation of a single ACV monophosphate molecule complementary to the opposite cytosine in the template. The complex then becomes irreversible and the ACV molecule cannot be excised by host cell exonucleases. Thus, the replicating DNA chain is terminated with the incorporated ACV monophosphate being unable to accept the subsequent incoming base in the chain in the absence of a second hydroxyl group corresponding to the 3' hydroxyl group of the natural nucleoside. ACV triphosphate has a higher affinity than the natural substrate for viral DNA polymerase, and thus it successfully competes for the enzyme resulting in preferential incorporation into the replicating DNA chain (Collins, 1996).

In a double-blind, placebo controlled study of ACV prophylaxis against HSV in 20 seropositive BMT patients, Saral *et al.,* (1981), found that 7/10 patients on the placebo developed culture positive HSV lesions, compared to 0/10 patients who received ACV. None of the patients had any evidence of drug related toxicity. Gluckman *et al.,* (1983), noted in their study of ACV prophylaxis in patients with high antibody titres to HSV, that protection against active infection was complete, and that some protective effect against CMV infection was also seen in that none of the patients given ACV had evidence of CMV infection. None of the patients on the ACV arm of the study developed CMV pneumonitis, compared with 3/18 patients on placebo who died of CMV pneumonitis in spite of treatment with intravenous ACV. The authors went on to suggest that some protection from graft verses host disease was also seen in patients given ACV prophylaxis. Wade *et al.,* (1983), have reported a more rapid marrow engraftment in patients given ACV prophylaxis. Although ACV is primarily of use in the treatment of HSV and VZV infection, recent studies have looked at the prophylactic use of high dose drug in the treatment of CMV disease in BMT patients (Prentice *et al.,* 1994).

Because of the high degree of specificity of ACV for HSV the possibility of resistant mutations arising was raised as being of concern, particularly in immunocompromised patients receiving long term treatment with the drug. This is discussed further in Chapter 6 of this thesis.

1.2.11.2 Penciclovir

Penciclovir (PCV; 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine is a potent and selective inhibitor of HSV and VZV in humans (Sutton & Boyd, 1993). PCV has virtually no activity against CMV. The advantage of penciclovir is that it has a longer half -life in cells than ACV, and reaches higher levels in infected cells. A disadvantage is that the triphosphate form of the drug is a much lower inhibitor of viral DNA polymerase than ACV. PCV is selectively monophosphorylated by HSV or VZV thymidine kinase, formation of di and triphosphate is catalysed by cellular kinases. PCV triphosphate competitively inhibits the viral DNA polymerase can be incorporated into the growing viral DNA chain, and because of the presence of the hydroxyl group on the acylic side chain, viral DNA extension may then occur for a limited extent after incorporation. In this aspect PCV differs from ACV triphosphate, which is an obligate chain terminator. An orally bioavailable derivative of penciclovir, called Famciclovir (Famvir), is now available. Famciclovir has a bioavailability of 77%, and is rapidly converted to its active form, penciclovir, by deactylation and oxidation. Isolates resistant to PCV can be selected by in vitro passage in the presence of drug (Weinberg *et al.,* 1992). Famvir is approved for the treatment of herpes zoster and recurrent genital HSV infection in immunocompetent hosts. Topical PCV for oral HSV and famciclovir for chronic hepatitis B infection are under study (Hammer and Inouye, 1996).

1.2.11.3 Valaciclovir

Valaciclovir (VACV: 2-[2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl-methothyl]ethyl-L-valinate hydrochloride, Valtrex) is the L-valyl ester of aciclovir and has a much improved bioavailability than ACV. VACV is different from previous candidate prodrugs, in that it is converted to ACV prior to anabolic phosphorylation, thus avoiding unwanted toxicity. The spectrum of activity of VACV is identical to that of ACV. VACV is an oral preparation that is rapidly absorbed from the gastrointestinal tract, resulting in a three to five fold increase in ACV bioavailability. VACV is effective in the treatment of recurrent genital HSV infection, and once daily dosing may provide effective prophylaxis against genital tract HSV in normal hosts (Reitano

et al., 1998). In the latter study, patients with less than ten episodes of recurrent infection were very effectively treated with 500mg of VACV once daily. However, VACV was found to be no better than 400mg, 2x daily, of ACV for the suppression of HSV recurrences in patients with more than ten episodes per year, and VACV was only equally as effective as the ACV regime if the patients were dosed twice daily. VACV is licensed for the treatment of shingles and is currently being evaluated in clinical trials for the treatment of CMV in Bone Marrow Transplant (BMT) and AIDS patients.

1.2.11.4 Foscarnet

Foscarnet (trisodium phosphonoformate, PFA, Foscavir) is a pyrophosphate analogue that differs from other major anti-herpesvirus agents in that it is not a nucleoside or nucleotide analogue. PFA has in vitro activity against HSV 1 and 2, VZV, CMV, EBV, HHV6, HIV and HBV. Foscarnet differs form the nucleoside analogue inhibitors in that it does not require intracellular metabolism for activation and in that it is a non-competitive inhibitor of viral DNA polymerases. PFA is not incorporated into the growing viral DNA chain. It blocks the pyrophosphate binding site on the viral DNA polymerase, thereby interfering with pyrophosphate exchange from dNTP moieties (Crumpacker, 1992). Oral bioavailability of the compound is as low as 10 to 20% and is therefore not suitable for oral administration. Since foscarnet does not require intracellular activation, resistance to this agent occurs exclusively at the level of the viral DNA polymerase. Clinical isolates of HSV, CMV and VZV resistant to foscarnet have been described, an d in vitro susceptibility testing of HSV strains derived from HIV infected patients has been shown to correlate with clinical success or failure (Safrin *et al.,* 1994). For these viruses, cidofovir is an important therapeutic option. Foscarnet is useful for the treatment of ACV resistant infection, although it is of little use if the resistance is due to mutations in the DNA polymerase gene because of cross-resistance at this locus.

1.2.11.5 Ganciclovir

Ganciclovir [9-(1,3-dihydroxy-propoxymethyl) guanine (GCV, Cytovene) is an acyclic nucleoside analogue of guanosine that is structurally similar to aciclovir, differing only in the presence of a hydroxy methyl group. This minor difference confers GCV with a markedly improved activity against CMV. GCV has activity against HSV types 1 and 2, VZV and EBV, but the clinical usefulness of GCV arises from its activity against CMV. Further discussion of the antiviral activity of GCV is therefore left till the section on CMV (1.3.15.2). It should be noted however, that HSV and VZV isolates that are resistant to ACV on the basis of thymidine kinase deficiency exhibit cross-resistance to GCV (Safrin *et al.,* 1991). HSV isolates resistant to ACV on the basis of mutations in the DNA polymerase may remain susceptible to GCV.

1.2.11.6 Cidofovir

Cidofovir {(S)-1- [3-hydroxy-2 (phosphonymethoxy)-propyl} cytosine, HPMPC, Vistide} is an acylic phosphonate nucleotide analogue drug. Cidofovir was developed primarily as an anti-CMV agent but has broad anti-herpesvirus activity. Cidofovir is taken up by both virally infected and uninfected cells and does not require the action of a virally induced kinase to be converted to its active form. Because of its structure, Cidofovir already exists in an essentially monophosphorylated form. Cellular enzymes convert it to the diphosphate form, which in turn is a competitive inhibitor of the viral DNA polymerase. Cidofovir can be administered intravenously, topically or by ocular implant. Cidofovir diphosphate has an intracellular half-life of more than 48 hours and this feature is of particular importance for the treatment of CMV. Cidofovir is currently under evaluation for the treatment of ACV resistant HSV infections, either by the intravenous route or topically (Snoeck *et al.,* 1994, Lalezari *et al.,* 1997), and for CMV infections in AIDS patients (Lalezari *et al.,* 1998).

Figure 1.1 Schematic diagram showing the mechanism of action of ACV in the HSV infected cell. To mimic a true nucleotide and thus to inhibit herpesvirus DNA replication ACV must be phosphorylated to its monophosphate form by HSV encoded TK, subsequent phosphorylation to the diphosphate and triphosphate forms is mediated by host cell enzymes. Limited phosphorylation in the uninfected cell does occur in the presence of high concentrations of ACV under the influence of a 5' nucleotidase. Host cell TK is incapable of phosphorylating ACV.

1.2.11 In Vitro Isolation of ACV Resistant Variants

Progeny virus from HSV cultured in the presence of ACV was found to be less sensitive to ACV than the input virus (Field *et al.,* 1980). HSV TK, unlike its cellular counterpart, recognises a number of synthetic substrates such as ACV and bromovinyldeoxyuridine. Deficiencies in the viral TK can, therefore, result in resistance to these drugs. The resistant viruses raised by Field *et al.,* (1980), were found to be either TK deficient (TK-), or had very low TK activity.

In addition to the TK deficient viruses, variants with resistance to phosphonoacetic acid were found to be cross-reactive to ACV. Since phosphonoacetic acid only inhibits viral DNA polymerase, it was concluded that mutations in the DNA polymerase gene also confer resistance to ACV (Schnipper and Crumpacker, 1980).

The DNA polymerase of herpes simplex type 1 (HSV pol) is a member of a large family of DNA polymerases which includes those from bacteria, bacteriophages, animal viruses and the replicative nuclear DNA polymerases of eukaryotes. As a component of the viral replicative machinary, it interacts with other essential viral replication proteins and is therefore the target for antiviral drugs. Several drug-resistance loci have been identified in the HSVpol gene, some of them occurring at amino acid residues which are highly conserved throughout the family of DNA polymerases. Point mutations generated by site directed mutagenesis in the highly conserved motif C, (885-GDTDS, which is essential for elongation activity), inactivate the polymerase elongation activity. However, in a study by Dorsky and Plourde, (1993), a virus with a serine to alanine mutation at amino acid 889 showed wild type elongation activity, but was highly resistant to both phosphonoacetic acid and ACV triphosphate.

DNA polymerase (DNA pol, encoded for by the UL30β genes) mutants are very difficult to generate in vitro, culture derived resistant variants are invariably TK deficient. Larder and Darby, (1984), transfected host cells with HSV TK so that the

gene product was constitutively expressed, and allowed viral TK to phosphorylate ACV. This system was used to generate mutations at the DNA pol locus. It is important to note that a functional polymerase is essential for virus replication, so mutations that lead to resistance have to be subtle, resulting in an altered substrate affinity with no loss of enzyme function.

The authors also generated an ACV resistant variant which expressed TK but which was unable to phosphorylate ACV. These are known as altered TK mutants (TK^{alt}). Variants with double mutations in the TK and DNA pol have also been generated (Larder and Darby, 1984).

Clinical isolates of TK deficient and TK low/altered mutants are seen occasionally in immunocompromised patients, but isolation of DNA pol mutants is very rare. Although mutants with deficiencies in TK co-exist with sensitive variants in the untreated population, and would be expected to be selected out under drug pressure, there is thought to be only a 5- 10% chance of resistance occurring in immunocompromised patients. TK deficient (TK-) mutants grow less well than wild type at peripheral sites and are less neurovirulent and reactivate less efficiently from latent infections of mouse sensory ganglia (Coen *et al.,* 1978, Field *et al.,* 1980, Tenser *et al.,* 1979).

1.2.1 Genotypic Variations Conferring Resistance

1.2.13.1 TK deficient Mutants

These variants which produce no TK seem to arise spontaneously. Kit *et al.,* (1987), sequenced a resistant HSV2 isolate from a patient on ACV therapy and identified nucleotide deletions in the repeat sequences of the TK gene at positions 217 and 439 which resulted in frameshifts and premature termination at amino acids 86 and 182 respectively.

1.2.13.2 TK altered Mutants

HSV TK is composed of two identical subunits, each consisting of 377 amino acids. The native protein has binding sites for both the natural substrate and for phosphate donor ATP. Sequence analysis of the nucleotides of three TK ^{alt} mutants found single base substitutions which were responsible for the phenotypic attenuation: at amino acid residues 168 and 176, resulting in coding changes from alanine to threonine and arginine to glutamine respectively. These mutants were named B3 and TR7. These alterations were found in a region of high homology between HSV 1 and 2, residues 161 - 193. Both mutants retained their ability to bind ATP but were resistant to ACV, suggesting that nucleotide binding is associated with this region (Collins and Darby, 1991).

A third mutant, S, was found to have a tyrosine substituted for cysteine at position 336. Although the substitution was not at the predicted N terminus ATP binding site the mutant still had a reduced binding affinity for ATP. Recent studies have also shown that there are a number of different populations of HSV TK mutants which co-exist in any infection, these mutants have varying degrees of TK activity caused by deletion, insertion and base substitution mutations. Table 6.2 (Chapter 6) gives a summary of the genotype changes in these mutants together with their TK phenotype (Sasadeusz *et al.,* 1997, Gaudreau 1998).

Table 1.1 Thymidine Kinase gene altered Mutants generated in vitro: amino acid changes and resulting phenotypic variation (Collins and Darby, 1991)

Mutant	amino acid residue	Functional change	Genotypic Changes
B3	168	TK ^{alt}	alanine to threonine
TR7	176	TK ^{alt}	arginine to glutamine
S	336	TK ^{alt}	cysteine to tyrosine
	223	TK ^{alt}	arginine to histidine

1.2.14 In vivo models for HSV resistance

Animal models of HSV form useful tools for the study of natural infection with replication at cutaneous sites, the peripheral nervous system and the establishment of latent infection in dorsal root ganglia.

The HSV (TK) is non-essential for viral growth in dividing cells in culture. However, in vivo failure to express the TK gene results in a dramatic reduction in neurovirulence. Mutant viruses also grow less well on the skin and are less virulent when injected directly into the brain. Reactivation from sensory ganglia has not been possible. Tenser *et al.*, (1981, 1987) showed that the TK deficient (TK-) viruses could not establish acute or latent infection in ganglia of mice after intraocular injection. There is some evidence that the TK mutants can be reactivated from latently infected tissues, but this is usually in mixed infections where it is possible that wild type viruses provide help for the mutants. TK altered mutants behave much like wild type viruses in that they produce lesions at peripheral sites, establish latency and can be reactivated (Tenser *et al.*, 1996).

DNA polymerase mutants replicate normally at peripheral sites, establish latency and reactivate as wild type viruses, but there is some evidence that CNS infection is reduced.

1.3 Human Cytomegalovirus

Cytomegaloviruses (CMVs) are ubiquitous and infect many species including humans, other primates, rats, guinea pigs and mice, some of which have formed useful animal models for certain aspects of human CMV (HCMV) infection. For the purposes of this thesis the role of human CMV alone will be discussed and will be referred to as CMV throughout, unless other animal cytomegaloviruses are under discussion.

CMVs are highly species specific and, like HSV, specific strains of HCMV have been shown to be linked with geographically isolated populations. As with other herpesvirus infections, CMV establishes a persistent infection. Reinfection with other strains can occur under certain circumstances and has been reported particularly frequently in the homosexual male population. In most patients the infection is subclinical, and this is often also the case in immunocompromised patients (Griffiths and Emery, 1997).

CMV can be propagated in vitro in cells of human origin and, in cells such as embryo derived fibroblasts, produces characteristic cell enlargement with intranuclear inclusions similar to those produced by HSV and VZV. Some years prior to the identification of CMV, cellular changes in tissues of patients with fatal infection led to the early use of the term cytomegalic inclusion disease (CID). Cytomegalic cells were observed in the parotid glands of dying infants. Histopathologic studies of salivary glands of guinea pigs and humans led to the probable causative agent to be referred to as the "salivary gland virus".

Farber and Wolbach, (1932), found typical cytomegalic inclusions in cells of 12% of 183 infants who had died of a variety of unrelated conditions and thus provided early evidence of a relatively common subclinical infection.

HCMV was isolated in the laboratory independently by Smith, (1956); Rowe *et al.,* (1956), and Weller *et al.,* (1957). The now commonly studied AD169 strain was found at this time. Each laboratory found a similar cytopathic effect (CPE), which was produced in human fibroblast cells, and characteristically showed foci containing large, refractile cells. The term cytomegalovirus was coined by Weller *et al.,* (1957), to reflect the cellular changes produced by the virus in tissue culture.

1.3.1 The CMV Genome

CMVs have the largest genome of any of the herpesviruses, at 230-240 kbp for HCMV and murine cytomegalovirus (MCMV) (Lakeman *et al.,* 1979). The genome

of the laboratory strain AD169 is divided into two unique regions named unique short (US) and unique long (UL). The US is bound by an inverted repeat IRS and a terminal repeat TRS, while UL is flanked by two terminal repeats IRL and TRL. Restriction mapping analysis of human CMV has revealed a complex arrangement of unique and inverted repeats, including the existence of four genome isomers similar to those found in HSV. The whole genome of AD169 has been sequenced and published (Chee *et al.,* 1990). Sequence data predicts 208 open reading frames (ORFs), with 202 predicted proteins, some of which are present as two copies because they map to repeat regions. Taking these repeats into account there are around 178 unique proteins known to date. AD169 is a strain that has been passaged in cell culture over many years and lacks some of the ORFs described in other CMV strains such as Towne and Toledo. Deletion mutants lacking 15 kb of the Us region can be successfully grown in cell culture suggesting that the cell adapted AD169 has lost some genetic information during adaptation to cell culture.

1.3.2 Replication of CMV

1.3.2.1 Gene expression

Three regions have an extremely high density of closely packed inverted and direct repeats, the DNA replication origin, ori-Lyt, and two transcriptional enhancers. Productive replication involves the co-ordinated expression of ordered sets of viral genes. Gene expression may be divided into sequentially expressed classes. Alpha (immediate early, IE), β1 and β 2 (delayed early, E) and γ1 and γ 2 (late) based on time of synthesis after infection. Thus the regulating cascade is similar to that of HSV and other herpesviruses (see Section 1.2.5).

The CMV replication cycle is slow compared to other herpesviruses. CMV requires 48 - 72 hours to produce progeny viruses. It has been suggested that the slow viral replication is either the result of a slow rate of viral DNA replication, or of an accumulation of viral proteins. Unlike other herpesviruses, CMV replication does not immediately shut off host cell metabolism. Instead, synthesis of cellular proteins, RNA and DNA is stimulated by CMV (Estes *et al.,* 1977; Furukawa *et al.,*

1975). It is thought that CMV DNA synthesis is dependent on cellular DNA synthesis (St. Jeor and Hutt, 1977). Indeed, HCMV and MCMV both replicate more rapidly in actively dividing cells and HCMV is inhibited by pre-treating cells with agents such as actinomycin D which reduce host cell metabolism.

HCMV shows a very restricted host range in cell culture, primary human cells permit viral replication, as do fibroblast cells from the skin or lung. Primary skin fibroblasts from chimpanzees are also fully permissive. It is interesting to note that a high level of replication is observed in fibroblasts that are transformed with human papillomavirus 16 E6/E7 genes that increase the rate of cell division.
This dependence of HCMV on the host cell, and the slow rate of replication has meant that work on viral mutants has progressed slowly. The initial events in the replication cycle of CMV are understood only in general terms. Ultrastructural studies have shown that attachment of the virus and penetration occurs at the cell surface and maturation occurs in the nucleus, similar to HSV-1 (Compton *et al.,* 1992). Attachment proceeds rapidly and efficiently in both permissive and non-permissive cells, suggesting that the receptor(s) for CMV are widely distributed on many different cell types (Smith, 1986; Waner, & Weller, 1974). The highly restricted host range of the human CMV is determined by a post-penetration block involving a restriction to virus gene expression (DeMarchi, 1983). Human CMV adsorption to cells is not competed for by any other human herpesviruses, suggesting it uses a distinct receptor(s). The nature of the human CMV receptors and viral envelope proteins that interact with cell surface proteins is beginning to be understood. From the work of Adlish *et al.,* (1990), it appears that cell surface proteins of 30 – 90 kd size may be involved as receptors.

It is thought that heparan sulphate is involved in the initial attachment of the virus to the cell surface. The viral envelope proteins that mediate binding to the cell surface remain uncharacterised, to date there is only indirect evidence for the interaction of gH with a 92.5-kd receptor (Keay *et al.,* 1989).

Attachment to the cell surface is followed by penetration. Human CMV has been shown to penetrate cells within 5 minutes of exposure, and it would appear that the penetration is pH independent, and occurs by direct fusion of the viral envelope with the plasma membrane rather than by endocytosis (Compton *et al.,* 1992). Viral nucleocapsids rapidly make their way to the nuclear pores and deliver viral DNA to the nucleus. The earliest classes of gene expression can be detected in 20 – 30 minutes post exposure to inoculum.

1.3.2.2. Regulation of Gene Expression

It is likely that host RNA polymerase II transcribes CMV DNA in the nucleus of the infected cells. One CMV gene, encoding the ie1 product has been shown to be transcribed by RNA polymerase II in vitro (Thomsen *et al.,* 1984). There is no evidence that this or any_other herpesvirus encodes its own RNA polymerase, although viral functions modulate the cellular transcription events during infection. Viral gene expression is also regulated by events at post-transcriptional, translational and post-translational levels (Mocarski, 1991). A total of five regions of gene expression have been mapped on the CMV genome US36 – 38, IE1 and IE2, TRS1-IRS1, UL69 and US3. The cascade of CMV protein synthesis consists of the sequential expression of α (immediate early, IE), delayed early (E or β), or late (L or γ) proteins. IE genes are transcribed within 2 hours of virus entry and the process is initiated by host cell RNA polymerase II that transcribes CMV DNA in the nucleus. In human CMV the principal alpha gene product is the IE1491aa major immediate early protein (Stinski, 1978). The major immediate early proteins (MIEPs) are encoded for by the UL122/123 genes, which also contain upstream enhancers. The second most abundant is the IE2579aa product, which is also a nuclear phosphoprotein. Also present, though less abundantly is the IE2425aa product. The IE1 and 2 proteins are involved in both positive and negative regulation of virus gene expression and are the most abundantly expressed_CMV genes (Cherrington *et al.,* 1991). Other genes that encode for regulatory proteins include the US3 gene, UL36/37 and the TRS1/IRS1 genes.

The expression of IE1 and IE2 is under the control of a strong transcriptional enhancer and has been referred to as the major IE gene promoter-enhancer or the CMV enhancer (Cherrington & Mocarski, 1989). It is part of a complex upstream regulatory region controlling the expression of IE1 and IE2.

The α gene expression is followed by expression of the early, or β genes, and the late or γ genes. In all herpesviruses, expression of β and γ genes requires the synthesis of functional alpha gene products. The early genes may be further divided into β1 and β2 genes. Transcription of β1 genes begins 4 -8 hours pi and is unaffected by inhibition of viral DNA replication. Beta-2 expression is also independent of viral DNA replication and is detected 8 - 24 hrs post infection (pi).

1.3.2.3 Early Events

The designation of a gene as beta or gamma has generally depended on the sensitivity of gene expression to inhibition by a DNA replication inhibitor with specificity for the viral DNA polymerase. Because the replication cycle is prolonged the inhibitors need to be present for up to 36 hours. It is thought that the transcriptional regulation of beta and gamma gene expression involves the IE2 gene products to acting alone or with IE1 491aa,_UL36, UL37, TRS1, IRS1 or possibly the US3 products, and that these transactivators mediate the regulatory cascade through a number of cellular transcription factors. It is not yet clear what promoter elements are required for the activation or repression of expression (Mocarski, 1996).

The beta gene products that have been mapped and whose regulation has been studied include several that are likely to be involved in DNA replication and metabolism.

1.3.2.4 Late Events

The late genes may also be divided into sub-groups γ1 and γ2. Gamma 1 transcripts are first detected 12 - 36 hrs pi and are made even in the presence of viral DNA

inhibitors. Gamma 2 transcripts appear 24 - 48 hrs pi and accumulation of proteins is strictly dependent on viral DNA replication. Many of the late genes of CMV encode structural proteins.

HCMV DNA replication is prolonged because of a protracted early phase of gene expression during which time replicative proteins such as DNA polymerase and single stranded (ss)- DNA binding proteins accumulate and cellular metabolism is stimulated. Lytic DNA replication requires a replication complex encoded by a number of essential genes and a defined initiation site or cis-acting element known as ori-Lyt. The human CMV genome is rich in direct and inverted repeats. The ori-Lyt origin of replication is located in the centre of the UL region of the genome, upstream of UL57. A replication complex of 11 essential gene products are required for ori-Lyt mediated CMV replication and consists of two sub-unit DNA polymerases (ppUL44 and pUL54), a helicase-primase complex (pUL70, pUL102, pUL105), and a single stranded DNA binding protein (pUL57) (Pari *et al.,* 1993). These genes and their functions are shown in Table 1.2.

Viral DNA synthesis proceeds through two peaks which occur at 18 - 24 and 60 - 80 hrs pi and coincide with peaks of cellular DNA replication. The CMV genome recirularises soon after infection and the replicating DNA lacks terminal fragments. This is called "endless" or "rolling circle" replication.

Table 1.2 Eleven loci are required for CMV replication.

DNA pol	UL54
Pol-associated protein	UL44
ssDNA binding protein	UL57
Helicase -primase	UL70, UL105, UL101-102
Transactivators	UL36-38, IRS1 (or TRS1)
Unknown functions	UL84, UL 112-113

1.3.2.5 Packaging and Egress

After circularisation concatamers are formed and genomic inversion occurs within the concatemers. The cleavage/ packaging process preferentially inserts the termini derived from the S derived regions rather than the L component into the maturing capsids. Completion of packaging is discerned by a "headful" mechanism i.e. when no more DNA can be packed into the capsid. It is thought that the UL89 encoded terminase is involved in the cleavage of concatemers into linear lengths of DNA which are then packed into the capsids.

The capsid may acquire its envelope either during passage through the nuclear membrane or loose and reacquire the envelope from endosomes during egress. The virions may be transported to the cell surface in vesicles via the golgi apparatus.

In addition to complete mature virions, two types of defective particles are also seen in vivo. Non-infectious enveloped particles (NIEPS) are morphologically indistinguishable from mature virions but lack genetic material. Also released are "dense bodies" which are made up of a mass of tegument protein consisting mainly of UL83 gene products (pp65).

1.3.5 CMV Proteins

The nomenclature for identified proteins is based on the 1993 International CMV Workshop whereby p indicates protein; gp indicates glycoprotein, and pp, phosphoprotein. This is followed by the genetic locus e.g.; gpUL55 (gB) is the glycoprotein B encoded by the 55th ORF in the unique long region. Figure 1.2 shows a diagrammatic representation of the genes of CMV and associated proteins.

1.3.5.1 Capsid Proteins

Much of the early characterisation of CMV capsid proteins was provided by Gibson, (1983). pUL86 is the major capsid protein antibodies against which cross-react with HSV-VP5 and which shares epitopes with the gag precursor p55 of HIV.

pUL46 is the minor capsid protein and shares homology with VP19c of HSV. pUL80a is the assemblin protein which is cleaved to produce a protease and an assembly protein. This process is very similar to that described for HSV where cleavage of the assembly protein is associated with DNA packaging of HSV.

1.3.5.2 Tegument Proteins

A large number of proteins, particularly the phosphoproteins, are located in the tegument region between the capsid and the envelope, and are easily recognised as structural proteins (Landini & Spaete, 1993). ppUL32 encodes for the basic phosphoprotein pp150 which is the major immunodominant protein. ppUL83 encodes for the lower matrix protein pp65 which is a major target for MHC Class 1 mediated cytotoxic T-cell killing. It is also found in peripheral mononuclear leukocytes (PML) and endothelial cells during CMV viraemia in immunocompromised patients. In addition to this, ppUL82 (pp71), ppUL99 (pp28), and ppUL56 are tegument proteins.

Opposite Page

Figure 1.2 Schematic representation of the genes of CMV and proteins which have been mapped to date. Colour coding is as follows: Gold = transactivators; Pink = genes/proteins involved in DNA replication; Green = capsid assembly; Orange = tegument; Pale Blue = envelope, Dark Blue = others. (Reproduced with permission from Professor PD Griffiths and Dr VC Emery).

HCMV
March '97
PG & VE
TRS1
US27
US28
UL4
US6 -
US11
US9
US11
US6
US3
US3
US2
IRS1
UL16
UL18
TRL
US
IRL
UL32
UL33
UL36
UL37
UL44
UL45
UL46
UL48
UL49
UL54
UL55
UL56
UL57
UL65
UL69
UL70
UL72
UL75
UL78
UL80A
UL82
UL82
UL83
UL83
UL84
UL85/6
UL89
UL97
UL98
UL99
UL100
UL101/102
UL105
UL110
UL112/113
UL114
UL115
UL122
UL123
UL127
UL
UL

1.3.5.3 *Envelope Proteins*

Some envelope proteins are named after the HSV proteins with which they share a degree of homology eg, gB, gH. Others are classified according to biochemical characteristics. One such molecule is the gpUL55 encoded glycoprotein B which has homology with gB of HSV. Several distinct antigenic domains have been identified on this molecule with at least two neutralising epitopes AD1 and AD2 (Tugizov *et al.,* 1994; Wagner *et al.,* 1992).

Neutralising activity in human sera is predominantly against the AD1 epitope (Britt *et al.,* 1990). The gB molecule has conformational domains, three of which are in the central part of the molecule and the fourth is located in the extracellular C terminal region of the protein. It is thought that genetic variation in the AD1 and AD2 regions may lead to antibody escape mutants. Using restriction enzyme mapping assays 12 unrelated strains of CMV have been identified and classified into four groups gB1-4.
Monoclonal antibodies with neutralising activity have been shown to block penetration of the virus rather than attachment to the cell. Recombinant gB can absorb most of the neutralising activity in human serum, leading to the conclusion that gB would be a prime candidate for a CMV vaccine.

gpUL75 (gH) contains only a single linear neutralising epitope, though two other antigenic sites have been located in the molecule. It is thought that this protein is involved in the binding of virus to the cell surface or in the process of internalisation. Outer envelope proteins include gL, integral membrane protein and glycoprotein complex II.

1.3.5.4 *Transactivator proteins*

pUL122/123 ORFs encompass the major immediate early (MIE) region. pUL82 is a structural phosphoprotein. pUL69 has an important role in the transition from early to late gene expression. It is functionally homologous to the immediate early protein ICP27 of HSV, which also has an essential viral regulatory function. However, the UL 69 protein cannot substitute for ICP27 in HSV infection, suggesting separate roles for these proteins in αand β herpesviruses. With ppUL82, pUL69 is necessary for high level activation of the CMV immediate early promoter enhancer element.

1.3.5.5 *Other Proteins*

gpUL 18 of CMV has been shown to have a significant homology to HLA Class I. It is known that CMV infection in fibroblasts results in the down-regulation of MHC Class I mature molecules even though Class I mRNA levels in the cell remain constant. It was therefore postulated that the protein products of UL18 sequestered β2-microglobulin in the cytoplasm, thereby preventing the expression of mature Class 1 molecules at the cell surface (Browne *et al.,* 1990). A monoclonal antibody against β2-microglobulin and pUL18 co-precipitates the two proteins when cells are co-infected with two vaccinia constructs, one expressing pUL18 and the other β2-microglobulin. However, studies using UL18 deletion mutants have shown that cellular Class 1 shut-down was the same as that seen with the wild type virus, so pUL18 alone is not directly involved in the down-regulation of Class 1 expression. Three other putative homologues of cellular genes have been described (UL33, US27 and US28). US28 encodes a chemokine receptor but does not transmit a signal, and may play a role in interaction between virus and monocytes and macrophages during infection (Noete *et al.,* 1993).

Human CMV DNA polymerase was first identified on the basis of its enzymatic activity, and has been studied as a potential target for antiviral agents (Huang *et al.,* 1975). The DNA polymerase of CMV is encoded by the UL54 gene, and is the ultimate target of the nucleoside analogue Ganciclovir (GCV). Point mutations in

this gene can confer resistance to this drug. CMV has no thymidine kinase similar to that of HSV, and genetic studies have identified the gene responsible for phosphorylating GCV in infected cells as UL97. UL97 encodes a protein kinase and marker transfer studies have indicated that there are two loci responsible for GCV resistance, one mapped to UL84 (DNA polymerase) and the second to UL97 (Sullivan *et al.,* 1992, Smith *et al.,* 1997). Functionally, UL97 is the equivalent of thymidine kinase (TK) of HSV and VZV in that it is responsible for phosphorylating nucleoside analogue antiviral drugs. However, unlike TK, the role of UL97 in the normal replication of CMV is not known. Interestingly, the UL97 homologue in HSV does not phosphorylate nucleoside analogue drugs and functions entirely as a protein kinase. Specific mutations in the UL97 gene have been found to confer resistance to GCV in laboratory and clinical strains of CMV (Wolf *et al.,* 1995).

The antiviral agent BCDRB has been used to elucidate the function of the product of the UL89 gene. BCDRB prevents cleavage of concatameric DNA intermediates to their monomeric units prior to virion packaging. Viruses that are resistant to this compound have mutations in UL89, leading to the conclusion that the UL89 gene product must be a component of the terminase complex (Krosky *et al.,* 1998).

1.3.6 Effects on the Host Cell

Unlike other herpesviruses, CMV stimulates host cell DNA, RNA and protein synthesis throughout infection. Many different cellular proteins, including enzymes, proto-oncogenes, heat shock proteins, transcription factors and cell surface adhesion proteins are induced by CMV infection. It is likely that the effect of CMV on host cell metabolism has an effect on the activation and replication of co-infecting viruses such as HIV, other herpesviruses and papovaviruses. Various members of the herpesvirus family have been implicated as causative agents in malignancy. The observation that CMV infection stimulates host cell metabolism is consistent with the role for CMV in altering cell growth (Pande *et al.,* 1990). The modulation mechanisms used to evade the host's immune responses are discussed later in Section 1.3.10.

1.3.7 *Primary Infection*

The clinical outcome of an acute or primary infection is highly dependent on the immune status of the patient. Seronegative pregnant women and their unborn children are at greatest risk for disease (Fowler *et al.,* 1992), as are solid organ transplant patients. (Grundy *et al.,* 1988). Of course, not all immunosuppressed patients with primary infection get disease, for instance, in the bone marrow transplant patient it is reactivation of latent virus that carries the highest risk of disease. In the renal transplant recipient, however, restriction enzyme analysis has shown that re-infection of seropositive patients with a different strain of CMV has a higher risk of disease than reactivation of latent virus (Grundy *et al.,* 1988). Likewise, HIV positive patients rarely experience primary infection their disease normally results from reactivation of latent virus or re-infection.

1.3.8 *Persistence and Latency*

Like all herpesviruses, CMV remains with its host for life after primary infection. In healthy individuals this poses no threat, but for patients who become immunosuppressed either through infection with HIV or through treatment strategies for transplant operations, reactivation of latent virus can cause life-threatening disease. It is not known whether the virus enters latency by depositing its genome in certain cell types or continues a persistent low level replication. Cell culture models of human and murine CMV suggest a cellular restriction on virus replication (Koszinowski *et al.,* 1990, Koffron *et al.,* 1998). The interaction of CMV with monocytes, macrophages and tissue histiocytes may be the key to virus persistence. Cell associated viraemia that occurs during disseminated CMV infection is associated to some degree with mononuclear phagocytes. In humans it has also been shown that stromal cells from bone marrow are permissive to CMV, but myeloid progenitors are restrictive and could be a site for latency. PCR methods have

identified a monocyte population in the peripheral blood of healthy carriers as a site of persistence (Taylor-Wiedeman *et al.,* 1991).

Viral genome has been detected in cultured granulocyte-macrophage lineage cells from which quiescent infection could be reactivated (Kondo *et al.,* 1996). Direct reactivation of latent infection from monocytes or granulocytes-macrophages has not been reported, but this population is still the major suspect reservoir of latent virus (Pollack *et al.,* 1997).

In the immunocompromised host reactivated and primary infection is associated with polymorphs, monocytes and endothelial cells. It is becoming apparent that the T lymphocyte is not the primary target for latency (Koffron *et al.,* 1998). Cellular and humoral immune responses are thought to be responsible for clearing acute infection and may perhaps play a role in the maintenance of latency (Koszinowski *et al.,* 1990; Rasmussen, 1990).

1.3.9 Immunity to CMV Infection

The immune response to CMV is complex because CMV itself is immunosuppressive and can interact with cells of the immune system.

The immune response to CMV infection has three aspects:

a) humoral immunity,

b) antibody dependent cell cytotoxicity, and

c) cell mediated immunity.

1.3.9.1 The Humoral Response

Part of the humoral response consists of neutralising antibodies which are induced by the envelope glycoproteins gB and gH. Studies have shown that in the immunocompetent patient antibodies to gB are regularly detected, but anti-gH responses are seen only immediately following a recent symptomatic CMV

seroconversion. In patients with AIDS anti-gB antibodies predominate and may be raised, but anti-gH antibody is very rarely seen.

The importance of the antibody response to CMV infection was first noted in neonates infected post-natally by blood transfusion, where maternal antibodies acquired transplacentally were found to significantly reduce mortality and morbidity in the premature infants. Early exposure to the virus can ameliorate the disease caused by reinfection in some patients eg, pregnant women seropositive before the start of pregnancy have a reduced risk of transmission to the neonate. CMV can be transmitted in the presence of acquired antibody but the manifestations of disease are ameliorated (Stagno *et al.,* 1977, 1980). In HLA matched, T-cell depleted allograft recipients of bone marrow, it is the seropositive donor marrow that gives a protective effect against severe CMV disease in seropositive recipients (Grob *et al.,* 1987). Seropositive patients who received a seronegative marrow were found to have a fifty- fold increased risk of fatal CMV infection. In the light of these findings, pooled human gamma globulin has been used in the prophylaxis regimen of BMT and renal transplant patients, though some have argued that the benefits are modest compared to the relative expense of the treatment.

1.3.9.2 Cell Mediated Immunity

In MCMV cell mediated immune clearance is performed largely by cytotoxic T cells (CTL) (Kurz *et al.,* 1997). These are cells which are either CD4+ or CD8+ and are MHC restricted. Natural killer cells (NK) are also important in controlling virus until specific immunity develops but are insufficient in themselves to eliminate virus.

CMV reactive cytotoxic T cells are also seen in humans (Borysiewicz *et al.,* 1988; Reusser *et al.,* 1991). Li *et al.,* (1994), have shown the presence of the MHC Class I restricted CTL response correlates with protection from CMV disease. Adoptive transfer of immunity using homologous CMV specific CTLs may be an effective means of therapy in post transplant CMV disease (Riddell *et al.,* 1992). These

authors have shown that of 14 patients with detectable levels of CMV specific CTLs after adoptive transfer, none developed disease. Much of the CTL response is directed against the structural protein pp65. Deletion mutants used to estimate the contribution of pp65 CTL to the total CMV response indicate that between 70 and 90% of all CTL recognising CMV infected target cells were pp65 specific. NK cell activity has also been demonstrated against CMV infected cells (Borysiewicz *et al.*, 1985).

Individuals with defects in their cell mediated immunity (CMI) are at high risk of CMV disease, implying a central role for CMI in the control of CMV infection. Adoptive transfer of CD4+ T cells from the spleen of immune mice has a modulatory effect on MCMV infection either by cellular cytotoxicity or by other helper mechanisms. Interestingly, it is the CD4+ cells, and not the Class I restricted CD8+ cells that are important in controlling viral replication in salivary glands and this control is influenced by levels of α interferon. The low level of MHC Class I expression in the salivary gland may limit the effectiveness of CD8+ cells.

Humans mount a cytotoxic T-cell response that can be Class I or II restricted. CMV IE1 gene products and late gene products produce specific CTLs that are Class I restricted. Although much of the CTL is reactive to pp65 and IE1 proteins, the IE products have a limiting effect on CTL killing. Other structural proteins, particularly the tegument proteins pp65 and pp150 may be processed and presented on the infected cell surface and recognised by CTLs (Riddell *et al.*, 1991). CTLs directed against these proteins can mediate protection during adoptive transfer. Although the α gene product forms the primary target to cytotoxic killing in MCMV infected mice, in human CMV limited presentation of this protein precludes specific T-cell killing. Thus, the major targets for CTLs may be the early, nuclear proteins and the late appearing structural and non-structural proteins.

1.3.10 *Immune evasion mechanisms*

Another important factor involved in virus clearance by CMI is the down-regulation of MHC Class I expression in cell infected by CMV. This down-regulation prevents the CD8+ CTLs from recognising the target cells. It has been proposed that recognition of CMV structural proteins evolved because this MHC shutdown occurs later in infection as a result of the US11 gene product, acting either alone, or in conjunction with US6 products. Also involved are the US2 and US3 gene products, US3 is an immediate early gene which encodes an endoplasmic reticulum resident glycoprotein which prevents the intracellular transport of MHC Class 1 molecules to the cell surface. The US3 gene product interacts with MHC molecules during the early stages of virus replication. The US2 gene product uses a similar mechanism to the US11 product. Newly synthesised MHC Class I molecules are bound and selectively transported from the endoplasmic reticulum to the cytosol to be deglycosylated and degraded by proteosomes (Jones *et al.,* 1996, Ahn *et al.,* 1996). The US6 gene product binds directly with the transporter associated with antigen processing (TAP) and MHC Class I complex. This mechanism of removing Class I molecules is similar to that seen in HSV. HSV1 and 2 interfere with virus specific CTL recognition by shutting off the MHC class 1 expression by overall diminished protein synthesis, and by the retention of the MHC proteins in the endoplasmic reticulum. York *et al.,* (1994) have shown that it is the ICP47 protein that is necessary for this retention. In CMV infection it is thought that it is the expression of the US11 product that inhibits the surface transport of the MHC class 1 molecule. Previous work had implicated the UL18 product, which exhibits a significant homology to MHC class 1, as playing a role in interfering with MHC class 1 transport and function, however, mutant CMV strains lacking the UL18 gene are unimpaired in their ability to shut down the synthesis of mature MHC Class1 heterodimers, suggesting that the UL18 gene product is not responsible for the virus specific decrease in Class 1 expression (Browne *et al.,* 1990, Wierzs *et al.,* 1996). As a consequence of this Class I down-regulation, cells should become susceptible to NK lysis due to the "missing self" hypothesis of Ljunggren & Karel (1990). Recent data has shown, however, that the UL18 gene product is able to act as a

broad spectrum NK decoy by binding to an NK-inhibitory receptor (Reyburn *et al.,* 1997). NK inhibitory receptors normally recognise MHC Class I molecules and protect the uninfected host from NK attack. UL18 is homologous to the MHC Class I heavy chain of higher organisms, binds to β2 microglobulin (Browne *et al.,* 1990), and can present endogenous peptides on the cell_surface (Chapman and Bjorkman, 1998). UL18 binds to the c-type lectin CD94 inhibitory receptor on human NK cells and prevents NK lysis of the CMV infected cell (Reyburn *et al.,* 1997).

1.3.11 Epidemiology of CMV

CMV is specific to the human host and can be transmitted by direct or indirect contact. Close contact is required for horizontal spread because of the labile nature of the virus. Virus has been found in, and therefore spread by, oropharyngeal secretions, urine, cervical and vaginal secretions, semen, breast milk, tears, and blood (Lang *et al.,* 1975, Reynolds *et al.,* 1973, Stagno , 1980). CMV is also transmitted vertically from mother to infant congenitally, perinatally and post-natally. Worldwide, between 2 - 10% of infants acquire infection by 12 months of age, and later childhood transmission occurs through close family contact. Although there is no evidence of racial susceptibility to infection, CMV infection is found at very high rates in densely populated areas of Africa, South East Asia, and the Middle East.

CMV persists as a latent infection following primary infection and, in the normal host, reactivation occurs from time to time. Reactivations are usually asymptomatic in the general population and allow the horizontal spread of CMV. In pregnant women reactivation of virus can lead to intrauterine transmission to the unborn child. Stagno *et al.,* (1984), have shown that the rate of primary infection in pregnant women is increased in lower socio-economic groups and that young children with subclinical infection are a major source of infection to pregnant women. Childcare workers have a 20 fold increased risk of acquiring CMV compared to control groups without exposure to young children. (Stagno *et al.,*

1984; Pass *et al.,* 1986). It is thought that transmission is mainly by the oral and respiratory routes.

Intrauterine infection probably occurs as a result of viraemia and placental infection. Around 1% of seropositive mothers and 40% of those with primary infection transmit CMV to the foetus. There is a high rate of reactivation of CMV in the breast and, as a result, transmission via breast milk is a significant source of infection in breastfed infants. Perinatal infection can also be acquired through infected genital secretions during childbirth. Post-natally, salivary transmission is especially common among children in nurseries and day care centres, and asymptomatically infected children may transmit infection to adult staff and parents as well as to other children.

Reinfection with other strains of CMV can also occur, and re-infection and reactivation can be distinguished by restriction enzyme analysis.

1.3.12 Sexual Transmission of CMV

This is an important route of transmission in adults. 3 - 35% of pregnant women attending sexually transmitted diseases (STD) clinics excrete virus from the genital tract. This figure may be unrepresentative of the general population though, and excretion rates in males are unknown (Stagno *et al.,* 1975). Sexual transmission may be linked to high numbers of seropositive men who have seronegative partners, the number of sexual partners and young age at first intercourse. Seropositivity in the presence of other STDs and the lack of barrier contraception are also associated with increased transmission. Chandler *et al.,* (1985) and Collier *et al.,* (1987), have shown a high prevalence of CMV in sexually active homosexual men. Reinfection of the genital tract is common in sexually active populations, and co-infections with multiple strains has been detected in semen of male homosexuals and other STD patients (Drew *et al.,* 1984; Spector *et al.,* 1984).

1.3.13 Infection in the Immunocompromised Host

Viral excretion is markedly increased in the immunocompromised host. Allograft recipients and AIDS patients often excrete CMV from multiple sites (Drew *et al.*, 1984; Spector *et al.*, 1984).

1.3.13.1 Infection in the solid organ transplant recipient

CMV disease is a frequent occurrence in solid organ transplant recipients and is linked with poor graft and patient survival. Pass *et al.*, (1978), have shown that virus excretion in seropositive renal transplant recipients approaches rates of 100%. In the renal transplant patient CMV is thought to directly influence renal dysfunction and graft rejection (Harmon *et al.*, 1982). Glomerulopathy or enlargement of endothelial cells has been associated with CMV viraemia, but whether or not CMV directly causes the glomerulopathy is not clear (Colvin *et al.*, 1983; Richardson *et al.*, 1981).

There is an increased incidence of disease in patients who are transplanted with cadaveric organs (20%) and therefore receiving anti-rejection therapy, especially those with an anti-lymphocytic effect, e.g., anti-thymocyte globulin (ATG), or the murine monoclonal antibody OKT3 (Dunn *et al.*, 1994). Patients transplanted with living related donor organs are less likely to have problems with rejection and therefore have a lowered risk of CMV disease (6.7%).

CMV disease in solid organ transplant patients is manifested in a wide spectrum of clinical events. Mild to moderate disease may include fever, malaise, lethargy and myalgia. It may also result in leucopenia, cough or abdominal pain from pulmonary or gastrointestinal (GI) involvement. In more severe cases, pneumonia or pneumonitis, GI haemorrhage, hepatitis, pancreatitis or retinitis may occur, resulting in respiratory or multi-organ failure. Recent studies have shown that the quantity of virus in the blood of liver transplant patients is significantly associated with disease (Cope *et al.*, 1997). Similarly, in renal transplant patients the degree of viruria is directly correlated with CMV disease (Cope *et al.*, 1997).

1.3.13.2 Infection in the bone marrow transplant patient

Bone marrow transplantation (BMT) is a treatment option which can potentially cure 30 - 60% of patients with haematological malignancies which cannot be cured with primary treatment alone. The main obstacle to greater success is transplant related complications, particularly pulmonary infection. Pneumonia occurs in approximately 50% of patients and is associated with a mortality of 30 - 90% (Krowka *et al.,* 1985, Ettinger *et al.,* 1991, Winston *et al.,* 1979). The risks of developing certain pulmonary complications depend upon the type of transplant, type of marrow donor, and the time of transplant. Historically, most BMTs since 1970 have been allogeneic (from HLA matched siblings), or syngeneic (from a twin). Since 1980 an increasing number of autologous grafts have been used, thus making it possible to increase the age limit of patients and to expand the use of BMT for the treatment of solid tumours. More recently, peripheral blood stem cells (PBSC) and cord blood cells have been used instead of and in conjunction with bone marrow.

Most transplants are performed for severe aplastic anaemia (SAA), acute myeloblastic or lymphoblastic leukaemia, chronic myelogenous leukaemia and Hodgkin's and non-Hodgkin's lymphomas. Patients usually undergo treatment for their disease prior to bone marrow transplant and this treatment may also predispose the patient to pulmonary complications. Unlike patients with malignancies, patients with SAA are not treated with total body irradiation (TBI) or chemotherapy, but they are often given prolonged courses of antibiotics and corticosteroids, and may be profoundly neutropaenic. Patients with acute leukaemias and lymphomas receive intensive multi-agent chemotherapy in order to achieve remission.

The exact mechanism of CMV induced pathogenesis in the bone marrow transplant (BMT) patient is poorly understood. It has been postulated that release of cytokines by stromal cells is caused by CMV infection and that the cytokines inhibit haematopoiesis (Apperley *et al.,* 1989). Disease in this patient group is seen in a variety of organs including the gastrointestinal tract, lung, eye, liver and the central

nervous system (Table 1.3). CMV pneumonitis is the most important cause of morbidity in the BMT patient, and is associated with a mortality of up to 80% in untreated patients. The precise role of the mechanism by which CMV causes pathology in the lung is not clear, but is thought to be immune mediated (Grundy *et al.,* 1987). Severe disease in the lung can arise from very limited focal CMV infection, suggesting that it is not the virus induced damage to the cells, but virus associated immune response, particularly lymphocyte infiltration and resulting inflammatory events in the lung that cause the pathology. Pneumonitis only occurs once the marrow has engrafted and cell numbers are increasing. This is in contrast to the situation in AIDS patients who rarely develop CMV pneumonitis, even though CMV is frequently found in their lungs. A declining immune system, and specifically low T- cell level may account for this difference.

1.3.13.3 CMV Infection in the HIV infected patient

Epidemiologic studies in HIV-infected haemophiliac patients have suggested that CMV seropositivity is associated with an increased risk for the development of AIDS, raising the possibility that CMV may influence the natural history of HIV infection, in addition to acting independently as an opportunistic pathogen. Although there is clear in vitro evidence of the interaction between HIV and CMV, direct in vivo evidence of the role of CMV as a co-factor in AIDS is still lacking (Barry *et al.,* 1990).

Nevertheless, CMV remains one of the most important opportunistic pathogens encountered by patients with AIDS. Up to 90% of patients with AIDS develop active CMV infection and severe disease (sight and/or life threatening) occurs in 20% - 40% of patients (Gallant *et al.,* 1992; Pertel *et al.,* 1992). Several aspects of the natural history of CMV contribute to its significance as an opportunistic pathogen; the near universal seropositivity of patients at risk of HIV infection, the high risk of re-infection, and an increased rate of survival from other infections which enables the AIDS patient to live for relatively long periods with a nearly absent cellular immune response (Gallant *et al.,* 1992).

1.3.13.4 The Impact of HAART on CMV Infection in the HIV Infected Patient

CMV end-organ disease is one of the most common serious complications of AIDS, primarily in those with CD4 cell counts of less than 50 cells/cubic millimetre. CMV retinitis was first reported as a complication of AIDS in 1982 by Holland *et al.,.*, and by 1984 ganciclovir was in widespread use for its treatment. In early reports, patients who survived longer than six months without specific CMV treatment became visually impaired or blind (Jabs *et al.,* 1989, Palestine *et al.,* 1984). Three antiviral drugs are currently available for the treatment of CMV retinitis: GCV, foscarnet, and cidofovir. Unfortunately, despite long-term suppressive therapy, most patients eventually experience a reactivation of CMV infection, which often results in further retinal necrosis. Reinduction of therapy with higher doses of the same drug or by switching drug regimens can control viral replication and cell necrosis temporarily, and complete blindness is now an infrequent complication of CMV retinitis_(Jacobson, 1997, Jabs *et al.,* 1989). Since 1993, a series of new strategies for the treatment of HIV infection have profoundly changed the outcome of a number of illnesses associated with AIDS, such as disseminated CMV disease, disseminated *Mycobacterium avium* complex infection, Kaposi's sarcoma and non-Hodgkin's lymphoma (Brodt *et al.,* 1997). In response to highly active antiretroviral treatment (HAART) including protease inhibitors, CD4+ lymphocyte counts can rise substantially even in patients with advanced HIV disease. However, in one study of twenty-one cases of CMV disease (Mallolas *et al.,* 1997), 19/21 episodes of CMV illness (initial or relapse) developed within two months of starting HAART. The authors identified two sub-populations among these patients: those developing CMV disease before increasing CD4+ counts in response to HAART, and those developing CMV disease despite significant increases in CD4 cell counts. The possible explanation for the development of disease in spite of increased CD4 lymphocyte levels was given as being a defect in the diversity of the repertoire of the reconstituted cells despite an improvement in the absolute cell numbers. The authors recommend that CMV prophylaxis should be maintained despite achieving an increase in CD4+ cell counts during the first few months after starting HAART.

In a recent study by Walsh *et al.,* (1998), the use of HIV protease inhibitors in combination with anti-retroviral therapy was associated with a marked increase in the survival of patients with CMV retinitis.

1.3.13.5 Infection in the congenitally infected neonate

The incidence of congenital infection ranges from 0.2 - 2.0% of live births in developed countries, and it is thought that 10 - 15% of infected infants suffer long-term neurologic sequalae. Some studies have suggested that a decrease in maternal lymphoproliferative responses during pregnancy could aid transmission from mother to foetus (Gertz *et al.,* 1987). Maternal immunity to CMV is also highly correlated with clinical outcome in the foetus. Women who are immune prior to conception rarely deliver infants with symptomatic infection, whereas infection following primary maternal infection ranges from 8 - 10% (Fowler *et al.,* 1992).

Table 1.3: Manifestation of CMV 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

1.3.14 Laboratory Diagnosis of CMV Infection

Urine, saliva, throat and genital swabs, cerebrospinal fluid (CSF), blood, bronchoalveolar lavage and biopsy tissue can be used with a variety of detection methods for the laboratory diagnosis of CMV infection. Urine and salivary samples are usually collected for diagnosis of congenitally or perinatally acquired infection. Patients undergoing solid organ or bone marrow transplant need to be monitored on a surveillance basis from two to four weeks prior to transplant onwards with blood

and urine samples, together with biopsies and BAL samples as indicated by the clinical progress of the patient.

In adults with mononucleosis or hepatitis, blood or urine samples are tested, as are pregnant women with symptoms of a possible CMV infection. An adequate test for asymptomatic women which could identify infants at risk of congenital or perinatal infection is not available and testing in this group of patients is not recommended (Griffiths and Emery, 1997).

1.3.14.1 Histopathology

CMV in biopsy material is characterised by typical "owl's eye" inclusions (Farber and Wolbach, 1932). They occur in kidney tubules, bile ducts, lung and liver parenchyma, gut, inner ear and the salivary glands. To a much lesser extent inclusions are also seen in the brain. Histological evidence of infection in specific organs is particularly important in the confirmation of clinical diagnosis and other laboratory results, none more so than in transplant patients where the association of CMV infection and the clinical picture is difficult to correlate.

1.3.14.2 Cell Culture

Cell culture has long been considered as the gold standard for the detection of CMV in a variety of samples, and continues to be the standard against which new techniques are compared. Clinical samples are inoculated onto monolayers of human fibroblast cells grown either in tubes or in multi-well plates. After adsorption, the cell sheet is washed and the culture medium is replenished. Cultures are incubated at 37°C and examined regularly by microscopy for the presence of typical focal cytopathic effect (CPE). Although occasionally urine from congenitally infected infants produces CPE more rapidly, normally CMV grows slowly and cultures need to be maintained for 21 - 28 days before a negative result can be reported. The cell culture method has a number of disadvantages, not least the delay in obtaining a result and a low negative predictive value.

1.3.14.3 Rapid Culture Methods

These assays, though less sensitive than conventional cell culture, have the advantage of providing rapid results. Two rapid assays were developed independently in the US and the UK. The US assay is known as the shell vial assay, (named after the container in which the assay is performed), and the UK assay is known as DEAFF test (Detection of Early Antigen Fluorescent Foci). In the DEAFF assay, fibroblast cells grown on slides are inoculated with sample potentially containing CMV. After incubation overnight the culture medium is removed and mouse monoclonal antibodies against the IE and early antigens are used to bind to viral antigens expressed in the cytoplasm. A second antibody, a goat anti-mouse, conjugated with FITC binds to the antigen-MAb complex and the presence of fluorescent foci detected by UV microscopy.

The shell vial assay has a sensitivity of 70 - 90% as compared with cell culture, and the DEAFF test has a sensitivity of 78% compared with 76% for conventional cell culture (Pillay *et al.,* 1991).

1.3.14.4 The Antigenemia Assay

Monoclonal antibodies against the tegument pp65 protein can be used to detect CMV directly in polymorphonuclear leucocytes (PMNLs) (Grefte *et al.,* 1992). The advantage of this method is that a result is normally available in around 5 hours and requires no culture or PCR facilities. The sensitivity of this assay is equivalent to that of conventional cell culture or rapid detection assays. The assay materials are cheaper than those for PCR, and commercial kits are beginning to become available, but the assay is laborious to perform and interpretation of results can be subjective. Another disadvantage of this method is that samples must be transported to the laboratory and processed immediately, stored material cannot be used. Detection of the pp65 protein is not always indicative of a productive infection, as phagocytosed protein from an earlier, cleared infection may lead to false positive results.

The antigenaemia assay is widely used to detect CMV infection and to monitor disease in solid organ and bone marrow transplant patients, (Boeckh *et al.,* 1994, 1996), though its use for disease prediction in HIV patients is not as well

documentated. The antigenaemia assay can be quantitated to some extent, (Gerna *et al.,* 1995), and in one study of 122 HIV positive patients with CD4 counts of <100 cells/ìl the assay was found to have a negative predictive value (NPV) of 95% for CMV disease, but a poor positive predictive value (PPV) of only 47%, with a sensitivity of 91%. A higher PPV of 63% could be achieved if a positive result was taken to be >5 positive cells per slide, but at the cost of a decreased NPV and sensitivity of 84% and 67% respectively (Boeckh *et al.,* 1996).

1.3.14.5 Polymerase Chain Reaction

The use of hybridisation assays to detect viral nucleic acids was reported in the early 1980s. Because the sensitivity of these assays was significantly lower than that of cell culture, these assays did not come into widespread use. The development of the polymerase chain reaction (PCR) in the mid-1980s led to its successful use in the laboratory diagnosis of many viruses. So successful is this technology and its applications that it is quite likely to supersede other methods for CMV detection.
The PCR method employs specific oligonucleotide sequences complementary to the target DNA sequence which act as primer. Primers are single stranded and normally between 15 and 30 base pairs (bp) in length. The primers bind to the target DNA and the sequence is amplified in the presence of an excess of dNTPs and a thermostable DNA polymerase enzyme, usually derived from the bacterium, *Thermophilus aquaticus* (Taq). After multiple cycles of denaturation, annealing and extension a product containing many million copies of the target sequence can be electrophoresed on agarose or other similar media and visualised, normally by staining with ethidium bromide.

The criticism that the extreme sensitivity of the PCR assay may detect latent virus in all seropositive transplant recipients has been addressed by Griffiths and Emery, (1997), with a number of suggestions for the use of PCR methodology:
A PCR assay in which a threshold value of 10 genomes per volume of sample is required to give a positive result, thus reducing the sensitivity of the assay to avoid

detection of very low level latent virus has been described by Kidd *et al.,* (1993). Detection of CMV DNA in plasma instead of PMNLs or whole blood has been used by Spector *et al.,* (1992), to monitor viraemia in AIDS patients. Since it is likely that latent virus exists in the cellular fraction of the blood, plasma DNAemia should reflect an active infection. A study comparing cell culture with plasma PCR showed that only 2 of 11 plasma PCR positive patients yielded infectious virus, but this result may be more of a reflection of the relative insensitivity of culture methods rather than a reflection of false positive PCR results (Spector *et al.,* (1992). Reverse Transcriptase PCR (RT-PCR) amplifies viral messenger RNA and so should detect only replicating virus.

CMV DNA may be amplified from whole blood or fractions thereof, urine, BAL, CSF, vitreous fluid. Using in situ PCR methods it may also be detected in sections of paraffin embedded tissues, or more conventionally, in homogenates of biopsy material. The target sequence amplified varies in different laboratories, with some laboratories using the IE genes and others preferring to use selected sequences from gB as the target. Detection of IE genes may reveal false positive results due to incomplete or abortive DNA replication. A comparison of three PCR targets, the IE, pp150 and gB was performed in thoracic transplant patients, and in the studies described in this thesis, the 149bp gB primer set was found to be the most sensitive predictor of disease.

1.3.14.6 Quantitation of CMV DNA

Although good positive predictive values can be achieved by using two or more consecutive qualitative PCR positive results, (and therapeutic guidelines can be developed from qualitative results in this manner), potentially more accurate prognostic information can be offered if the viral load in urine, blood and other tissues can be quantified. An advantage of a quantitative measure of virus load in the blood or other tissues is for the monitoring of the efficacy antiviral therapy.

A variety of methods for quantitating CMV have been described, including titration of infectious virus in cell culture and counting the number of positive cells per given number of neutrophils as described for the quantitative antigenemia test (Gerna *et al.,* 1995).

PCR can also be quantitated in a number of ways, including the production of a calibration curve using serial dilutions of a known amount of target. However, because of inter-reaction variation inherent in each PCR assay and in order to eliminate potential false negative results due to inhibitory material in the clinical samples, it is important to incorporate an internal standard in each tube.

1.3.14.7 Novel Methods for the Quantitation of CMV by PCR

The majority of quantitative PCR and quantitative reverse transcriptase PCR (QC RT-PCR) methods use an internal control that is co-amplified with the target sequence. The internal control can be produced in a number of ways: scrambling of the internal sequence, mutation of the target amplicon as described for the method used in this thesis or deletion or insertion of sequences into the target amplicon. PCR products can be quantitated in one of two ways, depending on the target of interest. Absolute quantitation requires the use of an internal control or standard curve run at the same time as the PCR reaction. This type of quantitation is most often required for the measurement of virus loads. Relative quantitation can be performed without an internal control, and is most often used to determine changes in the levels of expression of cell receptor molecules, cytokines or chemokines.

A novel method for real-time quantitative PCR employs the 5' nuclease assay (Holland *et al.,* 1991, Lee *et al.,* 1993, Livak *et al.,* 1995), with a non-extendable oligonucleotide probe. The probe is labelled with a reporter fluorescent dye {FAM (6 carboxyfluoroscein)} at the_5' end and a quencher fluorescent dye {TAMRA (6carboxy-tetramehtyl-rhodamine)} at the 3' end. When the probe is intact, the reporter dye emission is quenched due to the close proximity of the reporter and quencher dyes. During the extension phase of the PCR, however, the nucleolytic

activity of the DNA polymerase cleaves the hybridisation probe and releases the reporter dye from the probe. The resulting relative increase I reporter fluorescent emission is monitored in real time during PCR amplification using a sequence detector, the 7700 Sequence Detector (PE BioSystems, Foster City, CA). The sequence detector is a thermal cycler, laser, and a detection and software system that automates the 5' nuclease based detection and quantitation of nucleic acid sequences. Fluorescence intensity produced in each of 96 tubes is measured in real time. This assay has a number of advantages: there is a broad dynamic range of detection, normally from 1 to 10^{10} genome copies, there is no post-amplification analysis and this avoids contamination of future assays by amplicons. Using probes with other dyes it is possible to use this assay in a multiplex assay which may be adapted to screening for point mutations, or screening programmes. Because the assay measures real time amplification it also has the advantage of more accurately measuring the target amplification than agarose gel based methods and other end-point detection systems such as the Roche Amplicor system. It should be noted, however, that the use of this 5' nuclease assay (TaqMan) is highly restricted by patent_legislation and may not legally be used for the detection of CMV, and many other virus targets. In addition, the use of this technology for clinical diagnosis is also not currently licensed. Other PCR based assays for detecting CMV (and HSV) such as those employing the SYBR Green dyes that detect double-stranded DNA are similarly restricted at the present time.

The transcription based amplification systems that serve as the basis for continuous nucleic acid amplification systems (NASBA and 3SR) may also be used for the detection and quantification of CMV. DNA amplification requires two preliminary heat denaturing steps, and the product of the transcription amplification is single-stranded RNA which is then detected by hybridisation (van Gemen *et al.,* 1995).

1.3.15 Anti-viral Intervention for CMV Infection

1.3.15.1 Aciclovir

Although anti-herpes virus agents such as 5-iodo-2'-deoxyuridine and trifluridine were the first to be shown to have activity against HSV, they were limited because of their toxicity to topical use only. ACV was the first truly selective antiviral agent to be developed. The mechanism of action of ACV and its use in the treatment of HSV infection has been discussed earlier in Section 1.2.11.

CMV has been found to be relatively insensitive to ACV in vitro, with IC_{50} values in the range of 10 - 200μM (Collins and Darby, 1991). Nevertheless, ACV has been shown to be effective in the prophylaxis of CMV disease in profoundly immunosuppressed patients (Balfour *et al.,* 1989). The poor activity of ACV against CMV reflects the reduced ability of the virus to activate ACV in the absence of virus-specific TK. However, a protein kinase homologue, Ul97, encoded by CMV, does catalyse the phosphorylation of ACV.

In a study of 86 BMT patients who were seropositive for both CMV and HSV, ACV was given for HSV prophylaxis. A control group of 65 patients who were seropositive for CMV alone did not receive ACV. CMV disease occurred in 22% of the treated group compared with 38% in the untreated group. Survival within the first 100 days post-transplant was significantly higher among the ACV treated group ($p = 0.0002$). Thus, ACV prophylaxis reduced the risk of CMV disease or death by half within the first 100 days after transplant. (Meyers *et al.,* 1988). Other studies have also shown the benefits of ACV prophylaxis in the management of CMV infection in the BMT patient. (Saral *et al.,* 1981; Prentice *et al.,* 1983). High dose oral ACV has also been evaluated for use in the control of CMV in liver transplant patients (Mollison *et al.,* 1993), although in a controlled trial of low dose i.v.GCV verses high dose ACV, GCV was found to significantly more effective (Winston *et al.,* 1995).

1.3.15.2 Ganciclovir

Ganciclovir (GCV) is a guanosine analogue with significant activity against CMV, and is approved for the treatment of CMV retinitis. Like ACV, GCV is dependent on viral and cellular enzymes for its antiviral activity. In CMV infected cells GCV is monophosphorylated by the protein encoded by the UL97 gene of the virus. Cellular enzymes further phosphorylate the drug to its active triphosphate form. The viral DNA polymerase then incorporates the GCV-triphosphate into the viral DNA and DNA synthesis is inhibited. Strains of CMV resistant to GCV have been described in AIDS patients receiving maintenance therapy for retinitis (Spector *et al.,* 1996, Drew *et al.,* 1991). Most resistance is due to mutations in the UL97 gene, although occasional mutations in the DNA polymerase gene have also been observed (Smith *et al.,* 1997).

1.3.15.3 Foscarnet

Most anti-herpesvirus agents are nucleoside analogues, but Foscarnet is an exception. This drug is used to treat CMV retinitis in patients with AIDS (Jacobson *et al.,* 1994), and has also been used to treat some cases of severe mucocutaneous infections caused by ACV resistant HSV (Chatis *et al.,* 1989; Youle *et al.,* 1990; Safrin *et al.,* 1991).

Foscarnet, (phosphonoformic acid, PFA) is a pyrophosphate analogue which also has potent anti-CMV activity. PFA does not require intracellular activation, it inhibits viral replication by directly blocking the pyrophosphate binding site of the viral DNA polymerase. PFA inhibits a number of viruses, via the non-competitive inhibition of polymerases, probably at the pyrophosphate binding site. Widespread use PFA is limited by its poor oral bioavailability and inability to cross the blood-brain barrier. In addition, foscarnet is a nephrotoxic drug which is excreted through the kidneys.

1.3.15.4 Valaciclovir and famciclovir

A disadvantage of ACV is its limited oral bioavailability (15 - 21%) while that of penciclovir (PCV) is even lower (1.5%). Valaciclovir (L-valyl ester) and famciclovir (diacetyl derivative of the 6-deoxy PCV) are prodrugs of ACV and penciclovir respectively, designed to deliver higher levels of ACV while maintaining ACV's safety profile. Valaciclovir is cleaved by a valine esterase in the intestine and the liver to yield ACV and L-valine, this cleavage occurs rapidly and increases the bioavailability of ACV to 63%. Valaciclovir has recently been licensed for the treatment of herpes zoster in the UK, and clinical trials are underway for its use in CMV prophylaxis. The results of one such trial are discussed in Chapter 2 of this thesis.

1.3.15.5 Other antiviral agents

There are a number of other compounds beginning to be in use or under clinical trial for the treatment of CMV in the immunocompromised host. These include HPMPA and HPMPC (Cidofovir), Lobucovir, the anti-sense oligonucleotide ISIS2922, and benzimidazole (BCDRB). For a detailed review of currently licensed and drugs under evaluation see Johnson and Johnson, (1996). Although GCV, foscarnet and cidofovir are effective and routinely used for the treatment of CMV retinitis, none eliminates virus from the retina successfully, and chronic suppressive therapy is required. Chronic therapy is associated_with the development of resistance and resistant CMV is associated with poor response to therapy. Jabs *et al.,* (1998), have reported resistance to both foscarnet and cidofovir in a study of 122 patients with CMV retinitis and resistance to BCDRB has been reported to map to two open reading frames UL89 and UL56 (Krosky *et al.,* 1998).

1.3 Aims of the Thesis

The aim of this thesis was to study the role of CMV and HSV infection in two main groups of immunocompromised patients, namely patients with AIDS and those undergoing bone marrow transplantation.

In Chapter 2, the aim was to determine the efficacy of a novel drug, Valaciclovir, against CMV in patients at risk of developing CMV retinitis. This formed part of the virology sub-study for the ACTG 204 clinical trial for VACV. Qualitative PCR methods were used on longitudinal samples of blood and urine obtained from 14 centres world-wide. Statistical analysis was used to determine the risk factors for the development of end-organ CMV disease in all patients and to provide an assessment of antiviral therapies and any survival benefits of VACV in this group of patients.

The second aim was to use existing quantitative PCR methods to monitor fluctuations in CMV load in the blood of bone marrow transplant recipients. The relative contribution of virus load to the development of CMV disease could then be analysed together with previously identified risk factors for disease such as recipient seropositivity, graft verses host disease and genetic predisposition.

The qualitative PCR method used in this thesis for the amplification of the gB target sometime produces non-specific results when amplicons are analysed by agarose gel electrophoresis. In order to aid the clarification of these non-specific PCR results, a detection method using an oligonucleotide hybridisation probe conjugated to alkaline phosphatase was developed and evaluated.

Finally, routine surveillance of HSV isolates from both HIV infected patients and bone marrow transplant patients occasionally suggested the presence of ACV resistant mutants which were subjected to phenotypic and genotypic studies. The activities of the viral thymidine kinase and the viral DNA polymerase enzymes were

determined as part of a collaboration with the Wellcome laboratories at Beckenham, Kent. The TK gene for these ACV-resistant isolates was then sequenced in our laboratory. The data may add to the recently published data on ACV resistance and provide an insight into the increasing rate of ACV-resistant HSV infections in severely immunocompromised patients who often need to undergo extended periods of antiviral therapy with ACV.

Chapter 2

The Effect of Valaciclovir on CMV DNAemia and Viruria detected by PCR in Patients With Advanced HIV Disease.

2.1 Introduction

CMV causes severe disease in 20% - 40% of patients with AIDS (Gallant *et al.,* 1992, Pertell *et al.,* 1992). CMV end-organ disease, especially retinitis, is a common serious complication of HIV infection. Retinitis accounts for 80 - 90% of all disease, and causes retinal necrosis which may progress to blindness (Griffiths and Emery, 1997). Oesophagitis, encephalitis and polyradiculopathy represent the remaining 10% - 20% of disease manifestations in this patient group (Gallant *et al.,* 1992; Pertell *et al.,* 1992; Feinberg *et al.,* 1998). Disease normally occurs in profoundly immunocompromised patients with CD4 cell counts of less than 50 cell/mm^3. Treatment of established CMV disease in the eye requires life-long parenteral and/or intra ocular administration of virustatic agents such as ganciclovir, foscarnet and recently, cidofovir. These agents are both toxic and expensive and, despite treatment, progression of retinal lesions usually occurs.

High dose ACV has been shown to be effective at reducing CMV disease in bone marrow, renal and liver transplant recipients (Meyers *et al.,* 1988; Balfour *et al.,* 1989; Mollison *et al.,* 1993). In a study by Prentice *et al.,* (1994), high dose intravenous ACV followed by oral ACV improved survival and was of benefit in prophylaxis against the effects of CMV after bone marrow transplantation. In an open-controlled study by Meyers *et al.,* (1988), high dose intravenous (500mg/m^2, 3 times a day) ACV from 5 days to 30 days after BMT was found to prevent CMV infection, disease and pneumonia. Valaciclovir, which is a valine ester of aciclovir, is rapidly and almost completely converted to aciclovir after oral administration, achieving three to four-fold higher total plasma levels of drug than those achieved with ACV. Valaciclovir (VACV), 2 g four times a day (qds), results in a total exposure of ACV similar to that achieved by IV ACV dosing at 10mg/kg three times daily, which is sufficient to inhibit many clinical strains of CMV (Cole and Balfour,

1987). In a Phase I study of VACV in patients with advanced HIV disease, high dose ACV, 2000mg of VACV given orally four times a day, achieved levels of ACV in the plasma that were comparable to those achieved in BMT patients who were protected against CMV by high dose intravenous ACV (Jacobson *et al.,* 1994).

Oral prophylaxis for recurrent HSV infection is commonly used in patients with advanced HIV disease. Two prospective randomised studies of such patient populations have shown a survival advantage associated with high dose ACV compared to placebo, but no significant effect on the development of CMV disease (Youle *et al.,* 1994; Cooper *et al.,* 1993). In one study involving HIV infected patients treated with a combination of zidovudine and ACV showed that ACV had no effect on CMV positivity from urine samples using conventional cell culture methods (Drew *et al.,* 1995).

The aim of this chapter was to perform the virological analysis for CMV on patients recruited onto a larger ACTG 204 clinical trial for VACV. This was a randomised, double-blind, comparative study of VACV, 2g qid, and two doses of oral ACV for the prevention of end-organ CMV disease and mortality in patients with advanced HIV disease. A high dose of ACV, 800mg qid was the dose used in the placebo controlled trials that showed a survival benefit without affecting the incidence of CMV disease (Cooper *et al.,* 1993; Youle *et al.,* 1994). A lower dose of 400 mg bid was chosen to suppress HSV recurrences and thus to prevent possible unblinding. Antiviral effects were measured using qualitative PCR in samples of urine and blood. The PCR analysis presented in this chapter is that performed on samples from patients recruited in trial centres in Australia and in Europe. A similar cohort of patients was recruited in the USA, but these patients were monitored differently and are not included in this report.

2.2 Patients and Methods

2.2.1 Patients

Three hundred and ten patients were enrolled on the ACTG 204 virology sub-study from 15 European and Australian centres. All were HIV antibody positive, CMV antibody positive, had CD4 counts less than 100 cells/mm^3 at entry and had no evidence of CMV end-organ disease. Patients were examined by dilated indirect ophthalmoscopy to exclude asymptomatic CMV retinitis. Patients were excluded if they had chronic nausea or vomiting, ocular media opacity or necrotising retinopathy; were pregnant or breastfeeding; had a history of hypersensitivity to ACV or known lactose intolerance; had received immunomodulators within 30 days prior to entry; had previously received anti-CMV agents; or had any of the following laboratory values: absolute neutrophil count of <500 cell/mm^3; estimated creatinine clearance less than 50 ml/min, total bilirubin >5mg/dL, or serum aminotransferase greater than 5 times the upper limit of normal.

Power calculations were performed for the whole study (of which the virology sub-study was a part) so that a given percentage of the untreated group would reach a disease endpoint during the trial period. However, achieving sufficient power is often not possible for trials of this nature where suitable patients are very difficult to recruit in sufficient numbers. Therefore, opportunistic sample population was used for this sub-study on the advice of statisticians and confidence intervals for the statistical assays were used to determine the significance of the trial. If the confidence intervals had been found to be wide this would have indicated an insufficient patient sampling and more patients would need to be recruited onto the study. The limited number of investigators not blinded to the drug regime performed this ongoing assessment of the statistical power or confidence intervals achieved throughout the course of the study.

Samples of blood and urine were taken at baseline, weeks 4 and 8, then at 8 weekly intervals while patients remained on the study medication. No samples were taken after the patients had permanently stopped study medication.

Patients were randomised to receive oral prophylaxis with VACV 2g qid, or high dose ACV 800mg qid (3.2g/d), or low dose ACV, 400 mg twice daily (bid), in a 3:2:2 ratio. CD4 cell counts and HIV p24 antigen levels were measured every 24 weeks.

2.2.2 Evaluation of CMV Endpoints

Indirect fundoscopy was performed by ophthalmologists at 24 weeks, or when clinically indicated. Photographic evidence of CMV retinitis was held at an independent study centre in order to comply with the blinded protocol of the study.

Patients were evaluated at local centres at scheduled intervals and when symptoms appeared using standardised questionnaires. All data pertaining to disease other than ocular symptoms, including laboratory and radiological results were reviewed by an international panel of seven investigators who were blinded to the drug assignment. A designation of CMV endpoint was given where there was evidence of CMV viraemia, or if there was a positive culture result from any site, in conjunction with clinical and histological evidence of invasive disease.

2.2.2 Statistical Methods

Patients were analysed according to their initial treatment assignment, i.e. on an intention-to-treat basis. t-tests, Chi-squared tests or, where appropriate, Fisher's exact test were used to assess the significance of any differences observed. Time-to-event, or Kaplan-Meier analyses were also performed,

with significance assessed by log - rank tests. The Chi-squared test was used to measure the association between different groups of categorical variables. This test makes the assumption that at least 80% of the cells in a table should have expected values of 5 or more. When this assumption was not appropriate, Fisher's exact test was used. This is a hypothesis test that involves calculation of the probability associated with all 2 x 2 tables that have the same row and column totals as the observed data. This calculation is complicated and rarely performed manually. In the unpaired two sample t-test the assumption is that the variable is Normally distributed and the variances between two groups are the same. Differences between the means of the two groups are then tested. Under the null hypothesis that the population means in the two groups are the same, this difference will equal zero. Therefore, a test statistic is used which is based on the difference between the two sample means, and on the value of the difference in population means under the null hypothesis. In a one sample t-test we are interested in whether the mean of the unknown variable in the population differs from the hypothesised value. A test statistic is used which is based on the difference between the sample mean and the hypothesised value. The confidence interval provides a range of values in which we are certain the true population mean lies. Thus, information is obtained on the magnitude of the differences that are suggested by the data. The data allows us to calculate, usually, a 95% confidence interval for the true treatment effect of the therapeutic regime. These intervals have the property that they will contain the true value 95% of the time, therefore it can be concluded with reasonable certainty that the true effect is within this range, and this magnitude statement allows a clinical interpretation. The p value on its own does not allow a clinical interpretation, these values must be interpreted in association with confidence intervals.

2.3 *Laboratory Methods and Materials*

Whole blood samples were taken with and without anti-coagulant, in order to obtain serum and plasma samples, and for DNA extraction from heparinised

blood. Urine samples were also taken and tested by PCR without further manipulation. Serum, plasma and urine samples were divided into four aliquots and stored at -70° C. DNA from 200µl of whole, uncoagulated blood was extracted using commercially available ion exchange columns (Qiagen,Hilden, Germany). All samples were given numerical identification and were tested in a blinded fashion. All of the sample processing prior to PCR was performed at laboratories in the participating centres.

2.3.1 Oligonucleotide Primers for the Amplification of a Target Sequence of gB by Qualitative PCR (qPCR)

The region of CMV chosen to amplify using PCR was from the Hind III f fragment of the gene encoding for the envelope glycoprotein B.

A single round PCR reaction was optimised to detect 200 (500 for the quantitative PCR assay) genome copies per ml of CMV DNA from either urine or extracted blood. The sequences of primers used to amplify a 149 bp product from the gB region of CMV in both qualitative and quantitative PCR reactions were as follows:

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

and 5' d-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 *et al.,* 1989). The oligonucleotides were obtained HPLC purified from Cruachem (UK). The freeze dried primers were reconstituted in 100µl of sterile distilled water (sdw), and further diluted to give stocks of 1µg/µl, and final working stocks of 120ng/µl. This was done in order to prevent repeated freeze- thawing.

2.3.2 Optimisation of gB PCR

The PCR had been fully optimised for concentration of magnesium ions, primer concentrations and cycling conditions by Fox *et al.,* (1992). The optimum concentration of magnesium chloride in the reaction was 2mM $MgCl_2$, with 120 ng of each of the gB primers. These optimised components were used in a reaction mixture consisting of 10x PCR buffer (250mM Tris-HCl pH 8.4, 170mM ammonium sulphate, 100mM β -mercaptoethanol, 0.02% (w/v) gelatin), 200µM of each deoxynucleotide triphosphate (dNTP), and 2.5 units of DNA Taq polymerase enzyme (Bioline, UK).

The dNTP stock was made up as follows (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)

100µl of each stock dNTP plus 1200µl of sdw were mixed, aliquoted in small volumes and frozen at -20 °C, 3µl of which gave a final concentration of 200µM of each dNTP per 100µl PCR reaction.

Thus, a 100µl PCR reaction mixture per tube was made up as follows:

10µl	10x NH4 buffer
4µl	$MgCl_2$ (of 50mM stock)
3µl	dNTP mixture
1µl	primer gB1 (120ng)
1µl	primer gB2 (120ng)
0.5µl	*Taq* polymerase (2.5 units)
75µl	SDW
---	
total volume: 95µl	

The volume of distilled water in the reaction was adjusted according to the amount of target DNA to be added. The reaction mixture was overlaid with 100µl of mineral oil (molecular biology grade, Sigma) to minimise

contamination and prevent evaporation of the reaction mixture during cycling. A "master mix" was made up and aliquoted into the required number of reaction tubes in a "clean PCR room" using aerosol resistant tips, sterile gowns and gloves. Urine samples were used directly in the PCR reactions; blood samples were collected in preservative free heparin, and extracted prior to PCR using commercially available ion exchange columns (Qiagen, Germany). 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. In order to avoid false negative results due to the presence of inhibitors in the samples, 5μl of the first tube contents were then transferred to a second tube. This was referred to as a 1/20 dilution. 5μl SDW only was added to every tenth tube to act as a negative control. One tube was inoculated with 500 copies of DNA extracted from a plasmid containing the 149 bp sequence of gB to serve as a positive control. In addition to this, a number of tubes which had been pre-sealed in the "clean PCR room" were also included. This was done to facilitate the tracing of possible contamination. Only one PCR tube was open at any one time and gloves were changed frequently to minimise cross-contamination. The PCR reaction was performed in a Hybaid TR2 thermal cycler (Hybaid, UK) in a third designated room. The cycling conditions were designed to maximise DNA amplification of replicating virus without amplification of latent virus and to minimise non-specific bands. (Kidd *et al.*,, 1993).

Cycling conditions:

Stage 1: 1 cycle:	94°C 4 minutes
Stage 2 : 40 cycles:	94°C 30 seconds
	60°C 30 seconds
	72°C 30 seconds
Stage 3 : 1 cycle:	94°C 1 minute
	60°C 1 minute
	72°C 10 minutes

2.3.3 Visualisation of PCR Amplicons by Agarose Gel Electrophoresis

The PCR products from each experiment were visualised using agarose gel electrophoresis. Three grams of agarose were weighed into a Duran bottle and melted in 100ml of 0.5X TBE for three minutes in a 750W microwave oven at full power. The melted agarose was allowed to cool to below 60°C and 0.5µl of a 10 mg/ml ethidium bromide solution (Sigma) was added and the agarose was poured into an appropriate number of gel frames, each with three 12 toothed combs and left to set at room temperature. The combs were removed carefully and the gels were placed in an electrophoresis tank containing sufficient 0.5X TBE buffer. 20µl of each PCR reaction mixed with 5µl of loading buffer (15% ficoll, 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 0.5X TBE at 120V 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, pH adjusted to 8.3

2.4 Results

Table 2.1 shows the demographic characteristics of the patients from the European and Australian centres compared with the entire ACTG 204 study group. Samples from the European and Australian centres were processed at the Royal Free Hospital Medical School. Median age and CD4 + cell counts are shown for each study drug group. 94% - 96% of the study population in all the drug arms was male. Of the patients enrolled as the European and Australian cohort 131 received VACV, and 179 received ACV.

2.5.1 Baseline Characteristics

A total of 1839 blood samples and 1862 urine samples were tested by PCR. A median of 5 urine (range 1 - 15) and 5 blood (range 1 - 15) samples were obtained from each patient. Both blood and urine samples were available from 243 (78%) of the 310 patients on the substudy. Blood alone was available from 17 (5%) patients and urine alone from 27 (9%). Neither blood nor urine was available from 23 (7%) patients. These characteristics are shown in Figure 2.1. Baseline blood samples were available from 260 (84%) patients and urine from 270 (87%) patients.

Overall, 54/260 (21%) of all the baseline blood samples were PCR positive and 114/270 (42%) of all the baseline urine samples were positive by PCR. Of the 243 patients from whom both blood and urine samples were available, both samples were PCR- negative in 117 patients (48%). Samples from 77 (32%) patients were PCR-positive in urine only, 22 (9%) patients were PCR-positive in blood only. Samples from 27 (11%) patients were PCR-positive in both blood and urine. These data are summarised in Figure 2.2.

Of the 270 patients with baseline urine samples available, those who were PCR-positive had lower CD4 counts (median 29 cells/mm^3, range 0 - 95, n = 114) than those who were PCR- negative (median 36 cells/mm^3, range 0 - 100, n = 156: p = 0.04, Wilcoxon). Of the 260 patients with baseline blood samples available, those who were PCR-positive also had lower CD4 counts (median 23 cell/mm^3; range 0 - 100, n = 54) than those who were PCR-negative (median 35 cells/mm^3, range 0 -99, n = 206), although this comparison was not statistically significant (p = 0.11, Wilcoxon).

Table 2.1: ***Patients entered into the European and Australian Substudy : Comparison of Valaciclovir and*** **Aciclovir** ***arm***

Patient Characteristics	**Valaciclovir**	***Aciclovir***	**Overall**	**Whole ACTG 204 Population**
	(n = 131)	**(n = 179)**	**(n = 310)**	**(n = 1227)**
Male	96%	94%	95%	94%
Median Age (years)	38	37	38	37
Median CD4+ (cells/mm^3)	33	32	33	32
Median Karnofsky Score	90	90	90	90

Figure 2.1 Summary of the blood and urine samples that were available at baseline

Figure 2.2 CMV results for patients with both blood and urine samples available at baseline (n = 243)

2.5.2 *Disease Progression according to Baseline PCR Status*

Overall, in this substudy, 62 out of 310 patients (20%) developed disease, a Kaplan-Meier rate of 24% by day 784 post trial entry. The baseline PCR results were used to divide patients up into those who were PCR-positive or negative in urine, or those who were PCR-positive or negative in blood. The proportion of patients who remained free of CMV disease in these groups are shown in Figures 2.3 (blood) and 2.4 (urine), and illustrates the effect of treatment on the development of CMV disease, stratified according to PCR status in blood and urine at baseline. The greatest difference ($p = 0.002$, log rank test) was observed in patients who were initially PCR-positive in blood (Figure 2.3). A significant treatment effect was also seen ($p = 0.02$, log rank test) in patients who were PCR-negative in urine at baseline (Figure 2.4).

2.5.3 *Risk for Disease Progression*

Multivariate analysis of these results showed that individuals who were PCR-positive in either blood or urine at baseline experienced a faster progression to CMV disease than those who were PCR-negative. The time to CMV disease in patients with DNAemia at baseline was significantly ($p = 0.002$) shorter than in patients whose baseline blood samples were PCR-negative. Individuals who were PCR-positive in blood were 2.57 times more likely to develop disease at any time than those who were PCR negative in blood. Time to CMV disease was also significantly shorter, $p = 0.02$, in patients who were PCR- positive in urine compared to those who were negative in urine. Patients who were PCR-positive in urine were 1.94 times more likely to develop disease than those who were PCR-negative in urine (Table 2.3).

2.5.4 Disease Progression According to Treatment Allocation

The proportion of patients in each treatment arm who were PCR-positive in urine at baseline were similar: 53/116, (47%) for VACV, and 58/150 (41%) for ACV, but this was not the case for blood specimens. Of the 109 baseline blood specimens from patients allocated to the VACV arm, 15/107 (14%) were PCR-positive compared to 39/151 (26%) of blood specimens from patients allocated to the ACV arm. (Chi-squared test p = 0.03). Cox's proportional hazards methods were used to adjust for this imbalance. The Kaplan-Meier analysis shown in Figure 2.5 shows a significant (p = 0.002) decrease in the number of patients who were PCR-positive in blood and were treated with VACV who

who remained free of CMV disease during the study period compared to those on the combined ACV treatment arms. This effect was not observed (p = 0.94) in patients who were blood PCR-negative (Figure 2.6).

Patients who were urine PCR positive showed no significant difference in the proportion of patients who remained free of CMV disease (p = 0.48), whereas there was a significant decrease in the proportion of urine PCR negative who went on to develop disease (p = 0.02) (Figures 2.7 and 2.8).

No difference was noted in cumulative survival in patients allocated to either the VACV or the combined ACV arms. (P = 0.87, Figure 2.9). However, when survival was analysed according to baseline blood and urine PCR results, a significantly higher proportion (p = 0.0001) of patients who were blood PCR- negative at baseline survived to the end of the study period compared to patients who were blood PCR-positive (Figure 2.10). This effect was not seen with baseline urine PCR result (Figure 2.11).

Table 2.2 ***Proportions of Follow-up Blood or Urine Samples PCR-Positive According to Baseline PCR Results***

Sample	No Patients	Treatment Allocation	
		VACV	ACV
Baseline PCR-positive			
Blood	54	18/75 (24%)*	74/162 (46%)*
Urine	116	53/116 (47%)	58/150 (41%)
Baseline PCR-negative			
Blood	202	33/448 (7%)**	61/616 (10%)
Urine	154	32/312 (10%)†	95/468 (20%)†

VACV = valaciclovir * p = 0.001 (significant) ** p = >0.05

ACV = aciclovir

† p=<0.001

Figure 2.3: Kaplan-Meier plot showing the proportion of individuals free of CMV disease stratified according to baseline blood PCR status

Table 2.3 ***Relative Hazards (RH) and 95% confidence intervals (CI) from both univariate and multivariate analyses of progression to CMV Disease***

	Parameters in	Blood			Urine		
Model	**Model**	**RH**	**95% CI**	**p value**	**RH**	**95% CI**	**p value**
Univariate	PCR - positive	2.57	1.41 - 4.70	0.002	1.94	1.11 - 3.39	0.02
Multivariate (excluding interactions)	Valaciclovir	0.59	0.32 - 1.08	0.09	0.56	0.31 - 1.01	0.05
	PCR - positive	2.60	1.42 - 4.76	0.002	1.97	1.13 - 3.45	0.02
Multivariate (including interactions)	Valaciclovir	1.03	0.51 - 2.06	0.95	0.31	0.11 - 0.93	0.04
	PCR - positive	4.51	2.24 - 9.06	0.0001	1.53	0.79 - 2.96	0.21
	Valaciclovir plus PCR-positivity	0.08	0.01 - 0.67	0.02	2.48	0.67 - 9.19	0.18

Figure 2.4: Kaplan-Meier plot showing the proportion of individuals free of CMV disease stratified according to baseline urine PCR status

Figure 2.5: Kaplan-Meier plot showing the proportion of individuals free of CMV disease stratified according to treatment allocation - patients who were blood PCR +ve

Figure 2.6: Kaplan-Meier plot showing the proportion of individuals free of CMV disease stratified according to treatment allocation - patients who were blood PCR -ve

Figure 2.7: Kaplan-Meier plot showing the proportion of individuals free of CMV disease stratified according to treatment allocation - patients who were urine PCR +ve

Figure 2.8: Kaplan-Meier plot showing the proportion of individuals free of CMV disease stratified according to treatment allocation - patients who were urine PCR -ve

Figure 2.9: Kaplan-Meier plot showing survival stratified according to treatment allocation

Figure 2.10: Kaplan-Meier plot showing survival stratified according to baseline blood PCR status

Figure 2.11: Kaplan-Meier plot showing survival stratified according to baseline urine PCR status

2.5.5 *Disease Progression According to Treatment with or without VACV*

When the interaction between PCR positivity and treatment effect was taken into account, blood PCR-positive individuals who received ACV experienced a significantly faster progression to disease than those who also received ACV but were blood PCR-negative ($p = 0.0001$). Multivariate analysis showed that the disease risk associated with a positive blood PCR result was negated, however, in patients who received VACV ($p = 0.02$) (Table 2.3). Thus, the effect of blood PCR positivity is to place patients at a higher risk of disease, but this effect is negated if these patients were given VACV, i.e. the effect of VACV in blood PCR positive patients is to reduce the increased risk of disease conferred by their positive status. Urine PCR status was not significantly associated with progression to disease in this treatment-based analysis. (In order to clarify Table 2.3 it should be noted that the univariate model assesses the relative hazard associated with PCR seropositivity in isolation. The multivariate model excluding interactions assesses the effect of VACV and PCR positivity in multivariate analysis but without assuming interactions between these two factors, whereas the final multivariate model includes these interactions. It is important to note that the relative hazard for disease is significantly reduced if patients are receiving VACV and are also PCR positive in blood at baseline. The baseline group for all comparisons in this table are of patients who were PCR negative and receiving ACV).

2.5.6 *Effect of VACV on Follow-up Samples*

Figure 2.12 shows a comparison of the effect of VACV and ACV on follow-up samples from patients who were blood or urine positive at baseline. 14% of patients on the VACV arm were PCR-positive in blood at baseline compared to 26% on the ACV arm. In patients who were baseline PCR-positive in urine 47% received VACV and 41% received ACV. In patients who were PCR-positive in blood at baseline no significant

antiviral effect was observed with either arm of the study. When urine PCR-positivity was compared with the treatment arms, however, a sustained decline in the number of patients with PCR-positive results was seen in the VACV arm, whilst most of the patients on the combined ACV arm remained PCR positive throughout the study period (Figure2.12).

URINE

Figure 2.12 A comparison of the effect of VACV and ACV on follow-up samples from patients who were a) blood or b) urine positive at baseline

2.6 Discussion

PCR testing of whole blood and urine demonstrated that VACV had an antiviral effect on CMV in vivo. This anti-CMV effect correlated with a reduced incidence of CMV disease among patients randomised to valaciclovir. The strongest beneficial effect of VACV was seen in the subgroup of patients who were PCR positive in blood at trial entry. Multivariate analysis indicated that the risk factor of CMV DNAemia interacted with treatment with VACV with the result that the relative hazard for CMV disease was low (0.08), i.e. VACV abrogated the disease risk associated with CMV DNAemia. This is comparable to the situation in transplant patients treated pre-emptively with ganciclovir (Goodrich *et al.*,, 1991; Schmidt *et al.*,, 1991; Rubin *et al.*,, 1991). In contrast, no interaction between PCR positivity in urine and VACV treatment was observed. Valaciclovir had a significant effect in patients who were urine PCR negative at baseline, a feature which corresponds to prophylaxis with high dose ACV in transplant patients.

Patients who had baseline blood PCR-positive results progressed to CMV disease significantly faster than those who were negative at trial entry. These results indicate that the detection of CMV DNA in whole blood provides a proximal marker of CMV disease in patients with advanced HIV disease, and that VACV delayed the onset of CMV end-organ disease in patients with CMV DNAemia. The former is consistent with studies in transplant patients and similar data from bone marrow transplant recipients is presented in subsequent chapters of this thesis.

In this chapter the correlation between CMV disease and PCR results was primarily restricted to the presence of CMV in baseline samples, because treatment with VACV affected both the outcome measure (CMV disease) and the parameter being assayed (PCR result). However, the observation that the proportion of patients who continued to excrete virus decreased with VACV treatment would suggest that the drug reduces the actual viral load in the blood

of the patient with the resulting reduction of virus being excreted in urine. No qualitative effect on virus was seen in blood samples with either ACV or VACV. This study also found a trend for lower CD4 counts in patients who were viraemic, but the range of values suggest that CD4 counts alone do not provide a useful predictor of active CMV infection.

In other groups at risk of CMV disease peak virus load in blood and urine has been identified as an important determinant of CMV disease (Cope *et al.,* 1997, Cope *et al.,* 1997, Bowen *et al.,* 1996, Gor *et al.,* 1998). In subsequent studies quantitation of the virus loads of patients in this study univariate analysis showed_that elevated virus load in both blood and urine at baseline were associated with increased risk of CMV disease (blood relative hazard (RH) 1.47 per log increase, urine RH 1.49 per log increase). Elevated virus load at baseline was also shown to be significantly associated with shorter survival time (Emery *et al.,* 1998). After adjusting for treatment allocation and baseline CD4 counts, elevated viral load in blood and urine at baseline was still associated with a significantly shorter time to CMV disease. However, over the study period VACV significantly suppressed the viral load in patients who were PCR positive at baseline in either blood or urine when compared with the combined ACV arms. Increased viral loads over the study period were also found to be significantly associated with increased progression rates to CMV disease. In contrast, the effect of VACV became of borderline significance, suggesting that the major effect of VACV is mediated through effects of CMV DNA load in blood and urine.

If increased virus load in the blood and urine, and/or the length of a viraemic episode is critical for the development of CMV disease, more frequent sampling of blood of patients on treatment (and after treatment is discontinued) may provide prognostic information early enough for further intervention in patients for whom prophylaxis fails. The results of the quantitative analysis showed that the risk associated with becoming PCR positive in blood or urine is high (RH, 4.84 and 1.92 respectively) relative to

the risks associated with an increasing virus load (RH, 1.80 per log increase in blood and 1.38 per log increase in urine). In general however, although knowledge of changes in actual virus load aids understanding of the natural history of the CMV infection, at the practical level, qualitative PCR_data provides sufficient prognostic information for the screening of patients on antiviral therapy.

The results presented in this thesis show that there is a long time lag between CMV disease and PCR positivity and development of disease. Therefore, long-term follow-up using qualitative PCR to monitor patients at risk of developing disease on or off antiviral treatment may aid CMV disease management. Although in recent years highly active antiretroviral therapy (HAART) has significantly reduced the incidence of CMV disease, monitoring of patients by qualitative PCR may provide a low cost proximal marker for imminent possible failure of HAART.

The absence of an antiviral effect of ACV is consistent with the failure of ACV to prevent CMV end-organ disease in AIDS patients (Cooper *et al.,* 1993; Youle *et al.,* 1994). This is in contrast with the picture in bone marrow transplant recipients treated with high dose ACV (Meyers *et al.,* 1988; Prentice *et al.,* 1994), and in renal transplant patients (Balfour *et al.,* 1989). The role of ACV prophylaxis in bone marrow transplant patients is discussed elsewhere in this thesis. However, as the Phase I study results of Jacobson *et al.,* (1994), shows, it is likely that in the HIV patients insufficiently high levels of drug are achieved with high dose regimes of ACV. Indeed, a recent study of an ACV dose of >3200mg/day showed a significant survival benefit in HIV infection, but although HSV and VZV infections were decreased, there was no decrease in CMV disease (Ioannidis *et al.,* 1998). The considerably higher levels facilitated by VACV may produce similar benefits against CMV that are seen in the bone marrow transplant populations. It would appear, therefore, that prophylaxis with aciclovir is effective against CMV in the HIV

patient, but until the arrival of the prodrug, VACV, this effect was not seen in this immunocompromised population.

These results provide an insight into the natural history and pathogenesis of CMV disease among patients immunocompromised as a result of advanced HIV disease. Although this study did not include a placebo arm, ACV in both high and low dose has been shown to be ineffective at preventing CMV disease in two earlier double-blind, placebo-controlled randomised trials in AIDS patients (Cooper *et al.,* 1993, Youle *et al.,* 1994). It is, therefore, reasonable to conclude that the correlation of DNAemia with CMV disease progression provides an estimate of the natural history of CMV infection in this patient group. Of patients who were PCR positive in blood at trial entry, those who were on the ACV arms had a median incubation period for CMV disease of approximately one year, i.e. CMV DNAemia precedes CMV end-organ disease by a considerable length of time, thus allowing the planning of effective therapeutic interventions at an early stage. Monitoring at-risk patients by PCR may, therefore, be used to initiate effective prophylaxis and delay the development of end-organ disease.

Other studies in bone marrow transplant patients have shown that using PCR to monitor CMV DNAemia in blood has allowed the use of ganciclovir for shorter, and therefore, less toxic regimes with effective prevention or abrogation of CMV disease (Einsele *et al.,* 1995). Studies of oral ganciclovir in patients with AIDS (Spector *et al.,* 1996,) using culture of CMV from urine samples have shown significantly decreased risks ($p = 0.007$) of CMV disease in patients treated with oral GCV whether or not they received concomitant therapy with aciclovir (Relative risk: 0.57 in patients who did receive ACV, 0.42 in patients who did not receive concomitant ACV). When plasma PCR was used to monitor the efficacy of drug, of 481 plasmas tested, 250 (52%) were PCR positive, with a corresponding overall relative risk of CMV disease in patients receiving placebo who were PCR positive as opposed to PCR negative of 3.84. The relative risk (RR) of CMV disease for patients on oral

GCV therapy was 0.11 in patients who were PCR negative, compared to a RR of 0.62 for patients on GCV who were PCR positive at baseline. Thus, the risk of disease was more effectively reduced in patients given prophylaxis with GCV than in patients treated pre-emptively on the basis of positive PCR results. This is in contrast to the effect of VACV seen in this study where a consistent but greater effect of VACV was seen with patients who were blood PCR-positive.

In summary, the results of the study presented in this chapter have shown that PCR positivity in blood at baseline is significantly associated with an increased risk of disease. We have also shown that the greatest effect of VACV was seen in patients who were at the greatest risk of disease. Although this data did not show a significant benefit of VACV in patients who were PCR positive in urine only at baseline, VACV was found to have had a significant prophylactic effect in patients who were PCR negative in urine at baseline, a feature corresponding to high dose ACV prophylaxis in transplant patients.

Chapter 3

Development of a method to detect CMV amplicons using an oligonucleotide probe directly conjugated to alkaline phosphatase

3.1 Introduction

The polymerase chain reaction, (PCR), is one of the most powerful and widely used techniques for the identification and characterisation of viral infectious agents (Whitley and Lakeman, 1995; Garson *et al.,* 1994; Gravitt and Manos, 1992; Birkenmeyer and Mushahwar, 1994).

PCR protocols are normally optimised for required sensitivities and specificities by altering concentrations of reagents such as magnesium chloride and oligonucleotide primers, and by adjusting the cycling conditions of the reaction. For example, higher concentrations of magnesium chloride and lower annealing temperatures increase the sensitivity of PCR, but may also decrease the specificity, giving rise to amplification of non-specific products. Occasionally, the extraction procedure used for blood samples prior to PCR also results in amplification of non-specific products.

Amplicons may be visualised by electrophoresis through agarose gels stained with ethidium bromide. Alternatively, amplicons may be immobilised by capture onto a solid matrix and detected using oligonucleotide probes either following Southern blotting or using enzyme based assays where the biotinylated strands of the amplicons are bound to streptavidin coated wells. Agarose gel electrophoresis is a simple, rapid and inexpensive method for analysing large numbers of PCR reactions, and therefore is most frequently used for routine PCR analysis. A disadvantage of this method is that interpretation of results can be subjective, particularly at low levels of amplification where bands are faint, or when multiple bands are produced by non-specific amplification.

The PCR methodology used to amplify a 149 base pair target from the glycoprotein B gene of CMV has been described in Chapter 2. This protocol is highly specific and gives reproducible and clear results in the majority of samples tested. Occasionally, however, the PCR amplification results in multiple bands of amplicons when visualised on agarose gels and a more specific identification is necessary. The aim of

this chapter was to devise and evaluate a method for confirming specific amplification in clinical samples using an oligonucleotide probe directly conjugated to alkaline phosphatase. An oligonucleotide probe directly conjugated to alkaline phosphatase, (AP), was designed and evaluated for its utility for the detection of amplicons from a PCR reaction for the 149 bp fragment amplified from the glycoprotein B gene of CMV.

3.2 Methods

3.2.1 Streptavidin coating of Microtitre Plates

25µl of a 25µg/ml solution of streptavidin (Sigma), made up in Tris HCl pH 7.6 with 0.1% sodium azide, was added to each well of a 96 well Nunc Maxisorb U-well plate and incubated overnight at room temperature in a humidified chamber. The wells were washed twice with phosphate buffered saline (PBS) solution with 0.05% Tween 20 (BDH Laboratories), and then filled with 400µl of blocking solution consisting of 1% bovine serum albumin (Sigma, Factor V) in PBS solution with 0.1% sodium azide. The plates were incubated at room temperature for one hour and were then ready for use. The blocking solution was removed immediately prior to use. Multiple plates were coated in this way and stored at 4°C for up to six months. For later experiments commercially available streptavidin coated plates were used (StreptMax, Advanced Biotechnologies Ltd., Epsom, Surrey), which gave identical results to the plates coated as described above.

3.2.2 Polymerase Chain Reaction

The primers and other conditions for the PCR reaction were those described by Darlington *et al.,* (1991). The sequences of the primers are given in Chapter 2.3.1. In this study, the PCR was performed under identical conditions except that the reaction mix included a 1:1 mix of non-biotinylated GB1 primer and a GB1 primer that was labelled at its 5' terminus with biotin. The biotinylated primer was synthesised by Cruachem Ltd., UK. The typical reaction mix for each PCR tube was made up as follows:

10µl PCR buffer (Bioline UK)

4µl $MgCl_2$ (from 50mM stock)

3µl dNTP mix (200µM of each)

0.5 units of DNA Taq polymerase enzyme (Bioline, UK)

50 ng primer GB1 (non-biotinylated)

50ng primer GB1 (biotinylated)

100 ng primer GB2

volume made up to 95µl with sterile distilled water

Details of the PCR protocol are given in Chapter 2.3.2. All clinical samples were processed and amplified according to this protocol, using 5µl of extracted blood or 5µl of urine, both tested at neat and at a 1 in 20 dilution to overcome potential inhibition.

3.2.3 Detection of Amplicons

25µl of each undiluted amplicon was added to appropriate wells of the streptavidin coated plate and the plate was incubated at room temperature for 30 minutes. The wells were then washed three times with PBS-Tween after which 40µl of freshly prepared 0.15M sodium hydroxide was added to each well. After precisely 5 minutes at room temperature, the wells were washed four times as before. During this incubation period, the alkaline phosphatase coupled oligonucleotide probe (alkaline phosphatase-5'- GGTACTCGTAGGCCGAGTT(GC)/CCGGCGATGA) The AP-labelled probe (synthesised by R&D Systems, Oxford, UK.) was diluted in PBS containing 2% dried milk powder (Marvel), 20% sheep serum (SeraLab) and 0.5% Tween-20. 5µl of the diluted probe was added to each well, and the plate was sealed and incubated at 37°C for 30 - 45 minutes. The wells were washed 4 times as before and 50 µl of substrate solution (NADPH) was added to each well. The plate was incubated at room temperature and shielded from direct light for 20 minutes, after which 50 µl of amplifier (diaphorase and alcohol dehydrogenase) was added to the wells. The plate was incubated at room temperature again for 20 minutes for optimum colour development before the reaction was stopped with 50 µl of 2M sulphuric acid. The substrate, amplifier and sulphuric acid were supplied as an Ampak produced by Dako Diagnostics Ltd. The optical density of the reaction products was determined at 492nm using a conventional ELISA plate reader.

A diagrammatic representation of the amplification reaction is given in Figure 3.1.

3.2.4 Generation of a Standard Curve

Known copy numbers of the 149 bp sequence which had been cloned into a pUC18 plasmid vector were amplified using the PCR in order to generate a standard curve. 1, 2, 4, 5, 8, 16, 32, 64, 128, 256, 512, and 1000 genome copies were amplified and assayed using the oligonucleotide probe method. All assays were performed in triplicate, and repeated 3 - 4 times to determine inter-assay and intra-assay variability.

Figure 3.1: Diagrammatic representation of the amplification reaction used to detect hybridisation of the AP-labelled probe to the PCR products

3.3 Results

3.3.1 Determination of the negative cut-off value

One hundred and forty seven blood samples from organ transplant recipients in whom no active CMV replication had been identified post-transplant by PCR were used to determine the negative cut-off for the assay. The mean optical density (OD_{492}) for these samples (+/- 1 Standard Deviation, S.D.) was found to be 0.085 +/- 0.006. The results followed a normal distribution. Thus, samples known not to contain CMV DNA produced a consistently low OD_{492} under the assay conditions described. As a consequence of these data, the negative cut-off for the assay was set at 0.104 OD_{492} (mean + 3 S.D.), and this value was used in all subsequent analyses.

3.3.2 Generation of Standard Curve

The correlation of the OD_{492} with varying amounts of plasmid DNA with the gB 149 bp fragment from 1 to 1000 genome copies is shown in Table 3.1 and Figure 3.2. This method had a lower limit of detection of 4 genome copies of CMV DNA and reached a plateau at 600 genome copies. This lower limit for reproducible detection was comparable with detection by agarose gel electrophoresis, which had a lower limit of detection of 5 genome copies of target. Thus, the AP-conjugated probe method was not significantly more sensitive than conventional gel electrophoresis.

3.3.3 Assessment of Assay variability

Intra-assay variability was between 2 - 5%, however, the variability was higher between assays and ranged from 10 -20% depending upon the copy number of input DNA. A 15%-20% variability was observed with low copy numbers (< 10 genomes). In spite of the variability at the lower levels of detection, the OD_{492} for 5 copies of target (mean OD_{492} = 0.157) was always well above the negative cut-off value (Table 3.1).

Table 3.1 Generation of a calibration curve for the detection assay for CMV PCR amplicons. Relationship between input DNA of known copy number and optical density measured at 492nm

Number of CMV genome copies of input DNA	Optical Density at 492nm
1	0.073
2	0.089
4	0.139
5	0.157
8	0.175
16	0.370
32	0.657
64	1.015
128	1.729
256	2.600
512	3.430
1000	3.143

Figure 3.2 Standard curve relating the optical density at 492nm to the copy number of input DNA for the 149bp amplification of CMV gB sequence

3.3.4 Use of the assay to assess non-specific PCR results

One hundred and sixty-eight samples from the ACTG 204 study described in Chapter 2, which had yielded non-specific results using the standard PCR method were subjected to further analysis using the AP-labelled probe assay. Of 44 samples that gave a non-specific result on agarose gels at both neat and at a 1 in 20 dilution of sample, 68% were CMV DNA positive following hybridisation. In contrast, samples in which the undiluted samples yielded non-specific bands, but which were negative for CMV DNA at the 1/20 dilution (n = 101) gave only 33.6% positive results. Finally, in samples that were negative undiluted but gave non-specific bands at the 1 in 20 dilution (n = 23), the true positive rate was 21.7%. The mean OD_{492} for samples that were positive was 0.63, indicating that these amplicons contained a reasonable quantity of amplified CMV sequence. Overall, only 2% of samples yielded results that were within 4 standard deviations from the mean OD_{492} of the negative samples. These results are summarised in Table 3.2.

Table 3.2 Distribution of results using the alkaline phosphatase labelled oligonuceotide probe

Agarose gel Electrophoresis sample dilution		Number of Samples	AP Probe assay Results		No. of Samples	Mean OD_{492} (range)
			Sample dilution			
Neat	1/20		Neat	1/20		
NSB	NSB	44	Positive	Positive	8	1.414 (0.107 - 3.424)
			Positive	Negative	14	0.842 (0.105 - 2.900)
			Negative	Positive	6	0.626 (0.105 - 3.596)
			Negative	Negative	16	0.052 (0.001 - 0.104)
NSB	Negative	101	Positive	Positive	0	N/A
			Positive	Negative	34	0.564 (0.11 - 2.972)
			Negative	Positive	0	N/A
			Negative	Negative	67	0.029 (0.006 - 0.080)
Negative	NSB	23	Positive	Positive	0	N/A
			Positive	Negative	4	0.486 (0.116 - 0.863)
			Negative	Positive	2	0.131 (0.109 - 0.153)
			Negative	Negative	17	0.022 (0.002 - 0.047)

NSB = non-specific bands N/A = not applicable

3.4 Discussion

The sensitivity and specificity of the PCR depends on many variables including the reaction conditions, primer sequences, length of the amplified sequence and genetic composition of the sequence being amplified. The PCR protocol used in the assay described in this chapter for the detection of CMV DNA in clinical samples has been described by Kidd *et al.,* (1993); Darlington *et al.,* (1991) and Roy *et al.,* (1993). For the majority of samples, the assay produces a rapid and clear identification of CMV DNA in urine and blood samples. Occasionally, however, some material, particularly DNA extracted from blood samples using ion exchange columns, produce multiple non-specific bands when the amplicons are visualised on agarose gels. Non-specific binding (NSB) can also occur, though rarely, with urine samples and may occur at both neat and at 1 in 20 dilutions of sample. In these instances it is difficult to know whether the sample contained the target sequence, and further clarification is necessary to provide a definite result.

The method described in this chapter employs an oligonucleotide probe labelled at its 5' terminus with alkaline phosphatase which binds specifically to the biotinylated strand of the amplified CMV sequence. The results from the plasmid reconstruction experiments (Figure 3.2) demonstrated that this assay reproducibly detected amplicons derived from as little as four genome copies of target sequence. Importantly, the optical density obtained for either four or five genome copies of target was substantially higher than the mean OD_{492} obtained for clinical samples known not to contain CMV (Table 3.1).

The direct detection of amplicons using alkaline phosphatase conjugated probes has been described by Rasmussen *et al.,*, (1994); Cano *et al.,* 1993; Whitby and Garson, (1995), although the sensitivity in some cases has proved limiting, (Rasmussen *et al.,* 1994). This may be attributable to either a relatively insensitive PCR or to limitations within the detection method for the hybrid product. The amplification system used to

visualise the hybridisation of the AP-labelled probe involves the production of coloured formazan and leads to a 100-fold increased signal, thus allowing the reliable detection of as little as four genome copies of target DNA. The gel electrophoresis routinely used for the detection of amplicons requires a minimum of 5 - 10 copies of target to produce clearly visible products after ethidium bromide staining.

Although the dynamic range of this assay does not approach that of chemiluminescent detection of alkaline phosphatase conjugated oligonucleotides (Whitby and Garson, 1995), it does have the advantage of requiring no new instrumentation other than that used for routine enzyme linked immunoassays, while still maintaining sensitivity.

In order to evaluate the utility of this assay we analysed a series of clinical specimens that gave indeterminate results in a conventional gel electrophoresis detection system. The results confirmed that in the majority of cases, a finite result could be obtained using the alkaline phosphatase conjugated oligonucleotide probe assay. Sixty eight percent of the samples in which initial analysis had yielded non-specific bands at both neat and at a 1 in 20 dilution tested as positive with the conjugated probe method, whereas only 33.6% were true positives in the combination non-specific neat:negative 1 in 20, and only 21.7% true positive with the combination negative neat:non-specific at 1 in 20 dilution. Thus, the samples which were negative for CMV either undiluted or diluted 1 in 20 in the routine assay were more likely to be truly negative than samples giving non-specific bands at both concentrations of analyte.

The hybridisation efficiency of the oligonuceotide probe may be diminished to some extent due to the stearic hindrance effects of the large alkaline phosphatase molecule attached, but the hindrance is very effectively compensated for by the downstream amplification process. It is not always clear why non-specific amplification occurs with some clinical samples, but to some extent the problem has now been resolved by the use of better quality Taq DNA polymerase enzymes such as AmpliTaq Gold (Applied Biosystems Inc.). Prior to the development of better enzymes, confirmatory assays based on the hybridisation of amplicon specific probes such as the one

described in this chapter provided a rapid and reliable means by which non-specifically amplified products could be distinguished from true target. The cost of the alkaline phosphatase conjugated probe method compared with the newer Taq polymerase enzymes is high and this method is probably most suitable for samples which continue to be problematic even with these enzymes. The advantages of the method described in this chapter are that no radioisotopes or specialised detection equipment is required, the results are objective and available in three to four hours. This assay can be easily adapted for the purposes of detection of target sequence in other PCR applications, thus avoiding the need to sequence amplicons or confirm using restriction enzyme digestion.

PCR assays are frequently designed so that an internal human genomic DNA sequence is co-amplified as a control to show that the reaction proceeded efficiently. The assay described in this chapter did not use this internal control because this assay had been developed and validated for diagnostic use without this internal control. The samples tested for this study formed part of a larger study in which the samples were tested for the presence of CMV DNA using the diagnostic assay procedure.

Southern blotting was not used to confirm the binding of the probe in this assay, although it could have been used as an additional confirmation of the specificity of the binding. However, sequence comparison performed for other studies had shown no sequence homology with human genomic DNA in this region, indicating that this probe was highly specific for the sequence within the 149 bp amplicon. It is also likely that the Southern blotting would have been less sensitive than the signal amplification system used for this assay. The probe did not bind non-specifically in the large number of negative control samples obtained by gel electrophoresis, this confirms that hybridisation of the probe occurred specifically within the 149bp region of the gB sequence.

Experiments were performed to use this methodology for a quantitative PCR assay using a similar rationale to that described in Chapter 4. The probe was designed to

bind only to wild type DNA which would remain bound to the streptavidin coated plate as a whole fragment, while the control DNA digested with restriction enzyme would not be detected. Thus, the difference between the signals from the undigested amplicons and the digested amplicons bound and hybridised in two separate wells could be used to quantitate the amount of unknown DNA amplified. However, several problems were encountered with inter-assay variability and considerably more work is required to optimise this method.

Chapter 4

Longitudinal Fluctuations in Cytomegalovirus load in Bone Marrow Transplant Patients: Relationship between Peak Virus load, Donor/Recipient Serostatus, and CMV Disease

4.1 Introduction

Disorders which may be treated with bone marrow transplantation include acquired disorders such as malignant disease (acute lymphoblastic leukaemia, acute myeloid leukaemia, chronic myeloid leukaemia, Hodgkin's and non-Hodgkin's lymphoma, solid tumours and sever aplastic anaemia), and inherited disorders (thalassaemia major, sickle cell disease, inborn errors of metabolism, immunodeficiency syndromes (SCID) and Fanconi's anaemia).

Bone marrow transplantation is a treatment option which can potentially cure 30 - 60% of patients with haematological malignancies which cannot be cured with primary treatment alone. The main obstacle to greater success is transplant related complications, particularly pulmonary infection. Pneumonia occurs in approximately 50% of patients and is associated with a mortality of 30 - 90% (Krowka *et al.,* 1985; Ettinger *et al.,* 1991; Winston *et al.,* 1979). The risks of developing certain pulmonary complications depend upon the type of transplant, type of marrow donor, and the time of transplant. Historically, most BMTs since 1970 have been allogeneic (from HLA matched siblings), or syngeneic (from a twin). Since 1980 an increasing number of autologous (using the patient's own marrow, harvested during complete remission of malignancy), have been used, making it possible to increase the age limit of patients and to expand the use of BMT for the treatment of solid tumours. More recently, peripheral blood stem cells (PBSC) and cord blood cells have been used instead of and in conjunction with bone marrow.

Early complications after BMT include drug toxicity, mucositis, cystitis, cardiac and renal dysfunction, hepatic disease, graft failure, immunodeficiency, interstitial pneumonitis, acute graft verses host disease and infections. These symptoms may also manifest as late complications, together with endocrine dysfunction leading to impaired growth in children, neurological impairment, secondary malignancies, chronic graft verses host disease, cataracts and continued immunodeficiency.

CMV remains the most significant infectious cause of severe disease and death in recipients of autologous and allogeneic bone marrow (Meyers *et al.,* 1986; Wingard *et al.,* 1988). The most serious of the disease sequelae in this patient population is CMV interstitial pneumonitis (Neiman *et al.,* 1977), although CMV gastrointestinal tract disease, hepatitis, encephalitis, vasculitis, retinitis and suppression of marrow function can also occur (Prentice *et al.,* 1997). Risk factors for disease include the underlying condition for example, leukaemic patients are at higher risk for CMV disease than patients with aplastic anaemia, partly because of the more aggressive conditioning treatment given prior to transplant. Increasing age and type of transplant also influence disease risk. Other risk factors include receipt of marrow from a seropositive allogeneic donor, reactivation of recipient CMV strains and acute GvHD (Enright *et al.,*, 1993). Recipients of autologous marrow, or marrow from a syngeneic donor have lower risks of disease than patients who are given an allogeneic marrow. In the absence of antiviral intervention up to 50% of patients who experience reactivation of CMV develop CMV pneumonia (Meyers *et al.,*, 1990). In addition, ~ 30% of CMV seronegative patients will acquire primary infection if they receive marrow from a CMV-seropositive donor, and are subsequently at risk for CMV pneumonia. The mortality rate for untreated CMV pneumonia is approximately 85% (Meyers *et al.,* 1986). Since the pioneering work of Meyers and colleagues in Seattle (Meyers *et al.,* 1988; 1990), many studies using a variety of methods including conventional cell culture (Webster *et al.,* 1993), the antigenemia assay (Ehrnst *et al.,* 1993, Enright *et al.,* 1993), and the polymerase chain reaction (Jiwa *et al.,* 1989, Einsele *et al.,* 1991), have illustrated the frequency with which CMV reactivates post-transplant and have identified the clinical utility of monitoring patients prospectively. These assays have allowed patient management strategies to be developed so that pre-emptive therapy is now widely used. In addition, placebo controlled trials of ganciclovir (GCV) and *aciclovir* (ACV), have demonstrated that these drugs can decrease CMV infection when used prophylactically (Goodrich *et al.,* 1993; Prentice *et al.,* 1994; Winston *et al.,* 1993). Intravenous ACV, 500mg/m^2 three times a day given 5 days before until 30 days after transplant was shown to significantly reduce the risk of CMV infection, disease and pneumonia at 100 days post-transplant when compared with a no-treatment group (Meyers *et al.,* 1988). In a similar study,

Prentice *et al.,* (1994), confirmed that high dose intravenous ACV reduced the risk of CMV infection 7 months after transplant when compared with patients given low dose oral ACV primarily for HSV prophylaxis. Nevertheless, CMV continues to cause significant disease in bone marrow recipients, necessitating continued routine surveillance for CMV coupled with specific diagnostic testing in target organs such as lung, liver and gut. Despite the significant reduction in CMV disease during the first 100 days post transplant offered by prophylactic GCV strategies there is an increasing appreciation that late presentation of CMV disease can occur (> 100 days post transplant) which has a high mortality (Li *et al.,* 1994).

There are four approaches that can be used to prevent the development of CMV infection and disease:

Prophylaxis: Antiviral agents are administered to all BMT recipients at risk of developing CMV disease. Therapy is started when no signs of active infection are present.

Suppression: Antiviral agents are administered to BMT patients when CMV is detected from peripheral sites such as in urine or throat washings, but prior to the development of any clinical symptoms of CMV disease.

Pre-emptive therapy: Antiviral agents are given when CMV is detected systemically, such as in the blood or bronchoalveolar lavage (BAL), but prior to development of symptomatic CMV disease.

Treatment: Antiviral agents are given when clinical symptoms of established CMV disease are evident.

For each of the preventative strategies outlined above a number of drug regimens are available. The antiviral strategies employed in this study are discussed in section 4.5 of this chapter.

In other patient populations at risk of CMV disease, CMV load has been identified as another risk factor for CMV disease. Specifically, cross-sectional and longitudinal studies have indicated the importance of monitoring CMV load in liver transplant patients (Cope *et al.,* 1997), renal transplant recipients (Cope *et al.,* 1997) and HIV

infected patients (Bowen *et al.,* 1996). There is, however, a paucity of information on longitudinal fluctuations in CMV load in the BMT patient (Rawal *et al.,* 1994).
In this chapter the role of CMV load in the blood of BMT recipients and the relative significance of the previously identified risk factor of recipient CMV seropositivity was investigated. The relative contribution of other factors such as acute graft verses host disease, GCV treatment and T depletion to CMV disease are discussed in Chapter 5 of this thesis.

4.2 Patients

Patients undergoing bone marrow transplantation for a variety of conditions including leukemias, solid organ tumours and aplastic anaemia were monitored for CMV in blood by PCR prior to transplantation and then at approximately weekly intervals post transplant during hospital stay. Follow-up samples were available at fortnightly or monthly out-patient visits and then at longer intervals after the patient had been discharged from hospital. If the patient was re-admitted in case of infection or leukaemia relapse, weekly surveillance was resumed. Recipients of both autologous marrow and allografts were subjected to the same surveillance. Where clinically indicated, other samples such as BAL, biopsy tissues and throat washings were assessed for cytomegalovirus infection by conventional cell culture, detection of immediate-early antigen fluorescent foci (DEAFF), and histopathology. During the period between June 1992 and September 1995, one hundred and ten bone marrow transplant recipients were monitored prospectively for the presence of CMV in blood by qualitative PCR (qPCR) and any positive samples were subsequently subjected to quantitative-competitive PCR (QCPCR). All QCPCR assays were performed in a blinded fashion.

74/110 (67%) patients received allografts from either a sibling or an HLA matched volunteer, and 36/110 (33%) received autografts from marrow harvested during remission and before conditioning treatment prior to transplantation. The conditioning regimen included either cyclophosphamide (CY) and total body irradiation (TBI) or CY-busulfan (BU) for leukaemia, or CY and thoracoabdominal irradiation (TAI) for severe aplastic anaemia. All patients received broad spectrum antibiotics and amphotericin B as prophylaxis against bacterial and fungal infections. 73% of sibling donated allografts were T-cell depleted, and 93% of allografts from matched unrelated donors (MUD) were T-cell depleted (depletion range 40 - 98%). Allograft recipients were given GvHD prophylaxis with Cyclosporin A and methotrexate. Where acute-GvHD was apparent post-transplant methylpredinsolone was also used. Autografts were not T-cell depleted. The T-cell depletion was carried out in the laboratory using

CAMPATH 1 antibody. Some patients were also treated with anti-thymocyte globulin (ATG) in vivo depending on the condition requiring BMT. The age of the patients ranged between 6 to 42 years. Donor and recipient serostatus for CMV IgG was analysed using a specific radioimmunoassay as previously described (Berry *et al.,* 1986). Details of clinical symptoms, antiviral chemotherapy and other interventions together with laboratory results for haematological and biochemical investigations during the post- transplant period were obtained from the patients' clinical notes.

4.3 Definitions of CMV disease

4.3.1 Adapted Plotkin Scoring System for Scoring the Severity of CMV Disease

The Plotkin Scoring system for the severity of CMV disease was originally devised for renal transplant patients. Prior to the criteria for CMV disease devised by the 5th International Workshop on CMV Disease, an adapted version of the scoring system taken from Plotkin *et al.,*, (1991) was used to define the severity of disease in the bone marrow transplant patients. Clinical manifestations of CMV disease were defined as shown in Table 4.1.

4.3.2 Definition of CMV Disease According to Workshop on CMV Disease

Patients were classified as symptomatic if they had one or more of the symptoms defined by the 5th International Workshop on CMV Disease (Ljungman *et al.,* 1995). Case definitions were as follows:

Pneumonia: Clinical symptoms of pneumonia - radiographic and/or hypoxemia plus CMV in bronchoalveolar lavage (BAL) or lung biopsy.

Gastrointestinal disease: Gastrointestinal symptoms plus histological evidence of CMV from gut biopsies.

Hepatitis: A combination of abnormal liver function tests (minimum of twice the upper level of normal for 2 consecutive days) and histopathological changes consistent with viral hepatitis together with CMV in liver biopsy by culture, immunohistochemistry, in situ hybridization or PCR.

Neurological disease: symptoms of encephalitis, transverse myelitis or other signs of diffuse CNS disease together with detection of CMV in CSF by PCR, culture or DEAFF.

Retinitis: Typical retinal lesions diagnosed by an experienced ophthalmologist.

Table 4.1 Adapted Plotkin Scoring System for scoring the severity of CMV disease in BMT Patients. Points are given to define the severity of symptoms in relation to CMV infection

Clinical Manifestations	Points
Fever (≥ 38.3°C)	
Mild (2 - 4 days)	1
Moderate (5 - 20 days)	1
Severe (≥21 days)	3
Thrombocytopaenia (< 100 x 10^8/L)	1
Hepatitis	
Liver enzymes ≥ 2 x normal	1
Jaundice	3
Pneumonia associated with CMV	
Infiltrate on X- ray	1
Infiltrate and symptoms	2
Ventilator support	3
Gastrointestinal bleeding or ulceration (biopsy proven)	3
Central nervous system changes	
Lethargy	1
Stupor	2
Coma	3
Renal insufficiency	
Creatinine level 2 - 4 times above best pre-Tx level	1
Creatinine level > 4 times above best pre-Tx level	2
Nephrectomy, permanent dialysis	3
Arthritis or muscle wasting	2
Superinfection (bacterial, protozoal, fungal)	3
Graft verses host disease	2
Retinitis	3
Death	4

Adapted from Plotkin *et al.*, 1991

4.4 Methods

4.4.1 Qualitative PCR (qPCR)

Amplification of a 149 bp target sequence from the glycoprotein B region of the CMV genome was used to identify CMV infection in blood as described in Chapter 2.3. The qualitative PCR was optimised to reproducibly detect 200 genome copies per ml (ge/ml) of blood. The veracity of the CMV amplicons has previously been established (Kidd *et al.*,, 1993).

4.4.2 Phosphorylation of primer for QC-PCR

The gB2 primer used in the PCR was 5' phosphorylated as follows; 200 ng 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°C water bath for 30 min and the reaction was then stopped by placing the reaction mixture directly at –70°C until use. All reactions involving radiolabelled [γ^{32}P]ATP were carried out following strict departmental radiation safety measures including the disposal of all radioactive waste in designated areas.

4.4.3 Quantitative Competitive PCR (QCPCR)

Blood samples that contained CMV by qPCR were subsequently analysed by QCPCR as previously described by Fox *et al.,* 1991. Briefly, 5µl of DNA extract was co-amplified with a known copy number, (between 50 and 10,000 copies), of control DNA which comprised a 149 bp CMVsequence from AD169 identical to the wild type sequence except for the incorporation of a mutation by site directed mutagenesis (SDM) to produce a unique restriction site for *Hpa*1 enzyme. This control DNA was cloned into a pUC 13 vector and used to transform *E coli* cells to provide stocks of known copy numbers for use in the QC-PCR reaction.

Co-amplification of a known quantity of control sequence in each PCR mixture compensates for local variations in PCR tubes and identifies inhibition of the reaction by substances present in the sample. After co-amplification of target and control sequences in the PCR, the products were subjected to restriction enzyme digestion using *Hpa*I and the digested products separated using polyacrylamide gel electrophoresis (PAGE). The control sequence was digested into two (77 and 72 bp) fragments whilst the target amplicons remained undigested as a 149 bp product and migrated more slowly. The use of [γ^{32}P]ATP to label the gB2 5' OH allowed the detection of these bands by autoradiography and scanning densitometry.

The master reaction mixture per PCR reaction was as follows;

10µl	10x NH4 buffer
4µl	MgCl (50mM stock)
3µl	dNTP mixture (200µM each)
1µl	gB1 (120ng)
1µl	gB2 (120ng)
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 a 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, the PCR was repeated using higher or lower quantities of control DNA as appropriate. Where availability of material allowed, samples were analysed in triplicate to yield an accurate quantitation, aiming for an optimal ratio of target to

control between 40 to 60%. The PCR cycling conditions were identical to those detailed in Chapter 2.3 of this thesis

4.4.4 Digestion of Target/Control gB Amplicons

Following agarose gel electrophoresis of the QCPCR products 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 (Promega, 5 U/µl), 2µl of 10 x Buffer J (200mM Tris-HCl pH 7.4, 50mM $MgCl_2$, 500mM KCl) and 7.4µl of SDW to make up 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 5 µl of loading buffer (15% Ficoll, 0.25g bromophenol blue, both Sigma, UK) 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 45mA until the blue dye had run off the gel. The gels were then placed in 200ml of 1xTBE, containing 3µl of a 10 mg/ml ethidium bromide solution to enable visualisation of the DNA, and photographed using a UV transilluminator. The gels were wrapped in clingfilm and exposed to autoradiography film (Hyperfilm-MP, Amersham, UK) placed directly onto the gels in an X-ray cassette which was placed at -70°C for 1-24 hours depending on the specific activity of the radiolabel. Scanning densitometry with a Shimazdu CS 9001 PC dual wave scanning densitometer was used to obtain a target:control ratio from which the CMV genome copy number per ml of blood was calculated. The QCPCR was similarly optimised to detect 200 genome copies of viral DNA per ml of whole blood.

4.5 Antiviral Chemotherapy

Aciclovir, 10 mg/kg/tds during in-patient stay and 800mg qds po subsequently for 150 days post-transplant was administered prophylactically to all allograft recipients. Ganciclovir (induction dose: 5mg/kg 12 hourly, maintenance therapy at 6mg/kg daily dosage dependent on the patient's creatinine clearance), or foscarnet (60mg/kg/tds)

was given where clinically indicated, i.e. for symptoms of hypoxia, interstitial shadowing or persistent fever not resolved by antibiotics and antifungals, or the presence of CMV by the DEAFF test in bronchoalveolar lavage fluid (BAL). Since November 1994 GCV therapy could be initiated on the basis of two consecutive PCR positive results in blood. When administration of ganciclovir or foscarnet was contraindicated high dose aciclovir (800mg, 5 times a day) was used.

4.6 Statistical Methods

The significance of differences between maximum viral loads in groups was assessed by the Mann-Whitney test. Initially, univariate relationships between the development of CMV disease and the factors of interest were studied using Fisher's exact test or chi - squared test with continuity adjustment as appropriate (recipient serostatus), or Mann-Whitney U test (differences in viral load). Where significant, these relationships were then quantified using logistic regression (both univariate and multivariate), and the results used to generate an equation to determine the expected probability of being symptomatic at any given viral load. For these analyses, odds ratios quoted refer to a 0.25 $\log_{10}$ increase in viral load.

4.6 Results

One hundred and ten bone marrow transplant recipients were monitored for the presence of CMV infection in the blood during transplant work-up and post-transplant by qualitative PCR. DNAemia was detected in 49 patients (45%). All PCR positive samples were then subjected to quantification using a fully quantitative competitive PCR assay. A summary of type of transplant, donor/recipient serostatus for CMV, peak virus load, and symptomatology for patients who were PCR positive for CMV in blood is shown in Table 4.2. Overall, 15 patients experienced CMV disease that reached the internationally agreed criteria outlined in Section 4.3.2. As expected, CMV disease was seen more frequently in allogeneic marrow recipients (14/74) than in recipients of autologous marrow (1/36; $p = 0.03$). Individual longitudinal viral load profiles for a selection of patients are shown in Figure 4.1, panels a - p. The viral load profiles differed markedly between symptomatic (Figure 4.1 a-p) and asymptomatic individuals (Figure 4.1 panels q - t), with asymptomatic patients often exhibiting low viral loads which were only detected transiently. In contrast, symptomatic patients experienced higher viral loads that remained elevated for substantial periods of time. In some instances, for example patient 11, elevated virus load during surveillance correlated with onset of overt clinical symptoms. However, in the case of CMV pneumonitis the peak virus load frequently preceded the onset of disease. In the ten patients with pneumonitis, peak virus load preceded clinically apparent disease by a median of 57 days (range -279 to +73 days).

Following pages:

Figure 4.1 Longitudinal profiles of CMV load in the blood of patients with and without evidence of disease. Panels a - p show patients who experienced CMV disease. Panels q - v are examples of patients who did not have CMV disease. Periods of symptoms are indicated by the black bars and the coloured bars indicate time and types of antiviral interventions.

a

Transplant summary: Allograft from sibling,partial T-depletion.
ALL. Relapse 11/12 post transplant.

b

Transplant summary: MM kappa, partially T-depleted allograft for CMV IgG: R+D+

Transplant summary:received partially T-depleted marrow from sibling,CMV IgG D- /R+

d

Transplant summary: CGL, Philadelphia +. Received T-depleted marrow from M.U.D. CMV IgG + (R).
Induction with Alpha IFN pre-transplant.

e

Transplant summary: Pre-A.L.L. to Philadelphia + CGL. Given allograft from unrelated volunteer (MUD), T - depleted. Patient died 1 year post transplant

f

Transplant summary: A.L.L. Mismatched allograft DR6 (13) / DR6 (14) from sister. Partial T - depletion. CMV IgG + (R)

Transplant summary: C-ALL CMV IgG pos recipient. Age 3. Partially T-depleted marrow from HLA identical brother.

Transplant summary: A.L.L. CMV IgG D-R-, given allograft from HLA identical sister

Transplant summary: ALL, CMV IgG + (R), given non T - depleted allograft from fully matched sister.. CMV DNAemia before transplant.

j

Transplant summary: A.L.L., Received non- T depleted allograft from sister. CMV IgG + D&R

Transplant summary: AML (M5A), received allograft from sibling, R-D+ seroconverted for CMV IgG

L

Transplant summary: CML, R-D- , T-depleted allograft from matched brother.pre-tx pericardial TB, fungal infections in nails death due to GI bleed & respiratory and liver failure

m

Transplant summary: CALL, CMV D-R+, given allograft from brother. On blinded trial for VACV.

n

Transplant summary: AML, CMV IgG +, Autologous transplant. Relapse of leukemia 120 days post transplant

Transplant summary: A.L.L., partially T depleted marrow from sister. CMV IgG R+,D-

Transplant summary:AML. given autologous marrow and PBSC. CMV IgG positive

Transplant summary: Fanconi's anaemia, given HLA matched volunteer marrow. Age 7 Death due to intracerebral haemorrage and gvhd due to unrelated marrow graft

t
mild abdo pain
fever, cough
mild GvHD
5000
4000
3000
2000
1000
0
CMV genomes/ml (blood)
100
200
300
days post transplant
PATIENT AP
KEY
ACV 400 mg tds

An initial assessment of CMV disease was performed using the adapted Plotkin scoring system outlined in Section 4.3. Using this scoring system a greater number of patients were classified as having symptoms correlating with CMV infection than when the assessment was repeated using the criteria defined by International Workshop on CMV Disease. With the adapted Plotkin classification system symptoms such as persistent fever, elevated liver enzymes, abnormal chest X - rays and superinfection with other micro-organisms were allocated a severity score and individual scores were added up to give a final, "cumulative Plotkin Score". A list of the cumulative Plotkin Scores and symptoms that corresponded with this score are shown in Table 4.3. The cumulated Plotkin Scores were plotted against peak virus load in the 49 patients who were PCR positive (Figure 4.2). There was no significant correlation between the cumulative Plotkin Scores and an increased virus load. 26/49 (53.1%) of patients had CMV disease as defined by the Plotkin Scoring system, whereas 15/49 (30.6%) were classified as having CMV disease using the International CMV Workshop definitions outlined in Section 4.3.2. For the purposes of all further analyses, it was these stricter disease definitions that were used to define symptomatic patients. Thus, the term "symptomatic" throughout this chapter refers to patients defined using these latter criteria.

Opposite Page and following:

Table 4.2 Summary of the patients included in the study: type of transplant, donor:recipient serostatus for CMV, peak virus load and symptomatology

Patient No	Transplant	HCMV serostatus Donor	Recipient	Peak Virus load (log_{10})	HCMV Disease
1	allogeneic	-	+	5.2	Hepatitis,gastritis
2	allogeneic	+	+	4.5	Pneumonitis
3	allogeneic	+	-	3.7	Hepatitis , pneumonitis
4	allogeneic	+	+	4.1	Pneumonitis, gastritis
5	allogeneic	-	+	5.1	Pneumonitis
6	autologous	-	-	4.0	Pneumonitis, gastritis
7	allogeneic	-	+	5.8	Pneumonitis, gastritis
8	allogeneic	-	+	5.4	Pneumonitis
9	allogeneic	+	-	3.6	Pneumonitis
10	allogeneic	-	+	4.7	Gastritis
11	allogeneic	-	+	6.0	Retinitis,hepatitis
12	allogeneic	+	+	5.2	Pneumonitis
13	allogeneic	-	-	4.4	Pneumonitis
14	allogeneic	+	+	3.7	Pneumonitis
15	allogeneic	+	+	4.3	Pneumonitis
16	autologous	+	+	4.1	None
17	allogeneic	+	+	2.7	None
18	allogeneic	+	+	2.7	None
19	allogeneic	-	-	3.8	None
20	allogeneic	-	+	4.5	None
21	allogeneic	+	+	5.6	None
22	allogeneic	+	+	3.6	None
23	allogeneic	-	-	3.4	None
24	allogeneic	-	-	3.8	None
25	allogeniec	-	+	4.9	None

Table 4.2 (cont)

Patient No	Transplant	HCMV serostatus Donor	Recipient	Peak Virus load (log_{10})	HCMV Disease
26	allogeneic	-	-	3.9	None
27	allogeneic	-	+	5.0	None
28	autologous	-	-	3.8	None
29	autologous	-	-	3.5	None
30	allogeneic	-	-	3.5	None
31	autologous	-	-	3.5	None
32	allogeneic	+	+	2.7	None
33	allogeneic	-	-	3.9	None
34	allogeneic	+	-	3.4	None
35	autologous	+	+	2.7	None
36	allogeneic	+	-	4.5	None
37	allogeneic	-	-	4.1	None
38	autologous	+	+	4.7	None
39	allogeneic	+	+	4.6	None
40	autologous	-	-	2.7	None
41	autologous	+	+	3.9	None
42	autologous	-	-	4.2	None
43	autologous	+	+	4.0	None
44	autologous	-	-	3.5	None
45	allogeneic	+	-	4.3	None
46	allogeneic	+	+	3.9	None
47	autologous	+	+	2.7	None
48	allogeneic	-	+	2.7	None
49	allogeneic	+	+	4.2	None

Table 4.3 ***Severity of CMV disease in BMT patients as defined by the Plotkin Scoring System***

Patient No.	Type of Transplant	Peak Virus Load log_{10}	Symptoms	Plotkin Severity Score
1	allogeneic	5.2	hepatitis, gastritis, fungal infections, death	11
2	allogeneic	4.5	pneumonia,persistent fever, HSV lesions	5
3	allogeneic	3.7	hepatitis, pneumonia, fungal & bacteria infections	7
4	allogeneic	4.1	gastritis, fungaemia, impaired vision, rash, diarrhoea	16
5	allogeneic	5.1	pneumonia, cerebral lymphoma, persistent fever	9
6	autologous	4.0	pneumonia, gastritis, fungal infection	8
7	allogeneic	5.8	pneumonia, gastritis, fungal infection, mucositis, fevers, rash	16
8	allogeneic	5.4	pneumonia	9
9	allogeneic	3.6	pneumonia	10
10	allogeneic	4.7	gastritis	10
11	allogeneic	6.0	CMV retinitis, hepatitis	14
12	allogeneic	5.2	pneumonia	19
13	allogeneic	4.4	pneumonia	7
14	allogeneic	3.7	pneumonia	22
15	allogeneic	4.3	pneumonia	13
16	autologous	4.1	none	0
17	allogeneic	2.7	none	0
18	allogeneic	2.7	none	0
19	allogeneic	3.8	fever	1
20	allogeneic	4.5	fever	1
21	allogeneic	5.6	persistent fever, chest consolidation,CNS symptoms,fungal infections	10
22	allogeneic	3.6	none	0
23	allogeneic	3.4	none	0

Table 4.3

24	allogeneic	3.8	lung crepitations, raised LFTs, severe diarrhoea, rash, fevers	11
25	allogeneic	4.9	haematuria, CNS symptoms, severe fevers	8
26	allogeneic	3.9	diarrhoea, fever, haematuria	3
27	allogeneic	5.0	pneumonia, persistent fever,arthralgia, raised LFTs,viral haemorrhagic cystitis	12
28	autologous	3.8	cough	1
29	autologous	3.5	persistent fever, cough	3
30	allogeneic	3.5	none	0
31	allogeneic	3.5	none	0
32	allogeneic	2.7	none	0
33	allogeneic	3.9	rash, severe pyrexia, diarrhoea, gastritis	8
34	allogeneic	3.4	none	0
35	autologous	2.7	none	0
36	allogeneic	4.5	none	0
37	allogeneic	4.1	none	0
38	autologous	4.7	enlarged liver, high LFTs, cough, persistent fever	7
39	allogeneic	4.6	raised LFTs, jaundice, fevers, fits, coma	11
40	autologous	2.7	none	0
41	autologous	3.9	none	0
42	autologous	4.2	none	0
43	autologous	2.7	none	0
44	autologous	4.0	raised LFTs	1
45	autologous	3.5	none	0
46	allogeneic	4.3	fever, cough	3
47	allogeneic	3.7	none	0
48	allogeneic	3.9	fevers, cough	3
49	allogeneic	2.7	renal failure	3

Figure 4.2 Cumulated Plotkin Scores for Patients who were PCR-positive plotted against peak virus load during surveillance

In symptomatic patients the first blood PCR positive result occured at a median of 25 days post-transplant and preceded diagnosis of CMV disease by a median of 70 days. In asymptomatic patients the first blood PCR result was obtained at a median of 28 days post- transplant ($p = 0.26$ Mann Whitney U test). In order to determine whether viraemia or disease was different in recipients of allografts versus autografts, or whether there was a difference between recipients of sibling donated marrow or matched unrelated marrow, the time to first PCR positive, peak virus load and disease in the three groups was analysed. There was no significant difference observed between the first PCR positive and graft type ($p = 0.28$), nor was there a difference between peak virus load and graft type ($p = 0.264$). Patients who received autografts had a lower peak virus load than allograft recipients ($p = 0.04$, Mann Whitney U test). 3/9 recipients of matched unrelated marrow (MUD) developed disease, compared with 11/28 recipients of sibling donated marrow. Fisher's exact test showed no significant difference between the two groups. In contrast, 1/12 recipients of autografts developed disease, compared with 14/34 allograft recipients ($p = 0.05$).

The relationship between peak virus load and disease is shown in Figure 4.3. Viral load was significantly elevated in symptomatic patients (median 4.5 $\log_{10}$ genomes/ml blood; range 3.6 - 6.0) compared to asymptomatic patients (median 3.8 $\log_{10}$ genomes/ml blood range 2.7 - 5.6) ($p = 0.002$). Similar analysis using the total area under the viral load-time curve (AUC) and AUC per day also showed a significant difference between symptomatic and asymptomatic patients.

Figure 4.3 Relationship between peak virus load and symptomatology in recipients of bone marrow

Since the number of surveillance samples obtained from asymptomatic and symptomatic patients were similar, (median 23 per patient, range 1-46 for both groups, autografts and allografts), the relationship between peak virus load during surveillance and number of surveillance samples positive for CMV by PCR was investigated. These results are shown in Figure 4.4 and indicate that high viral loads correlated with a higher frequency of positive surveillance samples i.e., a greater overall viral load as defined by the total area under the viral load-time curve (AUC).

The risks for disease associated with specific CMV recipient-donor combinations in the study population on whom viral loads were measured are summarised in Tables 4.4 and 4.5.

Table 4.4 Median virus load in PCR positive patients sub-divided according to the recipient:donor serostatus for CMV IgG

CMV serostatus	Median virus load	Range
R- D-	3.8	2.7 - 4.4
R+ D-	5.0	2.7 - 6.0
R+ D+	3.95	2.7 - 5.6
R- D+	3.7	3.4 - 4.9

P = 0.007 (Krushal - Wallis)

Table 4.5 The relationship between recipient/ donor serostatus for CMV and the odds ratios for CMV disease in patients who were PCR positive in blood

Donor/ Recipient CMV serostatus	Odds Ratio	95% Confidence Interval	p - value
R - D -	1	-	-
R + D +	1.83	0.30 - 11.26	0.51
R - D +	3.67	0.35 - 38.03	0.28
R + D -	6.60	0.97 - 44.93	0.05

Compared to the R-D- group, disease was most common in patients who were seropositive recipients of seronegative donor marrow, with an odds ratio of 6.6. Comparable odds ratios were obtained when all 110 patients were subjected to similar analyses. The relationship between peak viral load and donor/recipient serostatus for CMV at the time of transplant was subsequently investigated. The results shown in Figure 4.5 and Table 4.4 illustrate that seropositive recipients of a seronegative marrow had a higher median viral load, (5.0 $\log_{10}$ genomes/ml, range 2.7 - 6.0) than all other recipient-donor combinations (R+D- vs all other combinations; $p = 0.007$ Krushal - Wallis). Analysis of viral load differences between individual groups showed that the viral loads in the R+D- group were significantly higher than viral loads in either the R-D- or R+D+ groups, but the difference between the R+D- and R-D+ did not reach statistical significance due to the small number of patients in the R-D+ group.

In PCR positive patients, the odds ratios for disease associated with elevated viral load, and reactivation of recipient cytomegalovirus strains were analysed using a

logistic regression model. The results of the univariate and multivariate analysis are summarised in Tables 4.6, and show that elevated virus load and receipt of a seronegative marrow by a seropositive recipient was significantly associated with risk of CMV disease (odds ratio: 1.43 per 0.25 log 10 increase in virus load, $p = 0.05$). In multivariate analysis, virus load remained a significant risk factor for disease ($p = 0.006$) after controlling for recipient serostatus, whereas the disease risk associated with R+D- serostatus was negated once the effect of virus load was controlled for ($p = 0.93$).

The logistic regression model was then used to produce an equation describing the probability of CMV disease with increasing viral load. A fitted viral load-disease probability curve was produced using this data (Figure 4.6), and the sensitivities and specificities of the model, together with the negative and positive predictive values are shown in Table 4.7. Odds ratios for CMV disease were calculated at virus load levels of 4.0 and 5.0 $\log_{10}$ genomes/ml in blood as 6.4 (95% confidence interval 1.5 - 27.4) and 10.66 (95% C I 1.8 - 60.5) respectively. Thus, at a peak blood viral load of 4.0 $\log_{10}$ ge/ml there was a 6.46 fold increased probability of disease, which increased to over 10 fold at a peak virus load of 5.0 $\log_{10}$ ge/ml.

Table 4.6 Univariate and Multivariate logistic regression analysis of viral load and recipient seropositivity as risk factors for CMV disease

Parameter	Univariate analysis			Multivariate analysis		
	odds ratio	95% C.I.	p value	odds ratio	95% C.I	p value
Viral load (per 0.25 $\log_{10}$ increase)	2.56	1.14 - 5.73	0.0006	2.68	1.02 - 7.05	0.003
Recipient seropositive	4.9	1.2 - 20.4	0.03	0.56	0.06 - 5.3	0.62

Table 4.7 Predictive values for CMV disease risk at different viral load strata calculated from the viral load figures shown in Table 4.2. Values were calculated for each viral load threshold relative to the total number of patients with disease rather than patients present within the viral load strata.

Viral load $\log_{10}$ (genomes/ ml)	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Odds Ratios (95% CI)
3.0	100	23.5	0.37	1.0	-
3.5	100	41.2	0.43	1.0	-
4.0	73.3	61.7	0.46	0.85	6.46 (1.5 - 27.4)
4.5	40.0	97.1	0.86	0.79	10.66 (1.8 - 60.5)

Figure 4.4 Correlation of the number of PCR positive samples and the peak virus load

Figure 4.5 Peak virus load in patients classified according to the CMV IgG status of the donors and recipients

Figure 4.6 Fitted viral load-disease probability curve produced using the data from the logistic regression analysis showing the probability of CMV disease with viral load

In order to determine if early institution of GCV, not based on PCR positivity, had an influence on the viral load-disease association, patients were divided into those who received GCV therapy and those who did not. Median peak virus load in patients treated with GCV was 1.0 $\log_{10}$ higher than in patients not exposed to GCV ($p = 0.0002$) (Figure 4.7).

Figure 4.7 Differences in the median peak virus load between patients who were or were not exposed to GCV

4.7 Discussion

In this chapter quantitative PCR analysis was used to monitor fluctuations in cytomegalovirus load following bone marrow transplantation and to relate viral loads with symptomatology and donor/recipient CMV serostatus. The results demonstrate the central role of elevated viral load in the pathogenesis of CMV in this patient group. Thus, the median peak viral load in symptomatic patients was 0.7 $\log_{10}$ higher than the viral load in patients who remained asymptomatic ($p<0.002$) and was significantly elevated in CMV seropositive recipients of seronegative donor marrow ($p= 0.007$). Consistent with previous studies at our centre the R+D- sub-group of patients were most at risk of CMV disease (Grob *et al.,* 1987). The higher incidence of disease in this group may be attributed to the effect of T-cell depletion and corresponding lack of CMV specific T-cell responses from the donor marrow. In contrast, peak viral loads between the remaining recipient-donor combinations were comparable (ca. 3.7 $\log_{10}$ genomes/ml) including patients acquiring primary infection from exogenous sources (R-D-). It should be noted that although seronegative patients were given CMV screened blood products, filtered products were not used on any patient in this cohort. Some patients may also have been given packed red cells and platelet transfusions during the early stages post-transplant. Eleven seronegative patients experienced a primary infection which was confirmed by subsequent seroconversion. This relatively high number of patients with a primary infection may be explained by insensitive antibody screening assays which may have missed some patients who were previously seropositive but had low levels of antibody, or by infection post-transplant which may have been acquired from family and other hospital visitors. Moreover, CMV specific RNA has been reported in up to 40% of CMV-seronegative, healthy blood donors (Zhang *et al.,* 1995), suggesting either an abortive infection or an insensitive antibody assay. The observation that the R+D+ patient group had a lower incidence of CMV disease and lower viral loads than the R+D- group could be explained by adoptive transfer of immunity from the donor resulting in reduced viral replication (Grob *et al.,* 1987, Wimperis *et al.,* 1986). Such adoptive transfer of immunity has been demonstrated for tetanus toxoid and hepatitis B vaccines in the bone marrow transplant population (Riddell *et al.,* 1992).

This observation that there was no difference in virus load or disease incidence between patients who received marrow from relatives or unrelated donors is consistent with a recent report by Hongeng *et al.,* (1997). These authors suggest that this may be due to improved HLA matching, prevention of GvHD and the effect of better antiviral prophylaxis and pre-emptive treatment.

Since both recipient seropositivity and elevated viral load were associated with CMV disease multivariate logistic regression analysis was used to determine the relative contribution of both of these parameters in the 49 PCR positive patients. These analyses revealed that viral load was the major factor associated with disease and that the statistical significance of recipient serostatus was abolished ($p = 0.93$) after controlling for the effect of viral load.

Prophylaxis and pre-emptive therapy are now used widely in the management of the bone marrow transplant recipient (Einsele *et al.,* 1995, Schmidt *et al.,* 1991, Goodrich *et al.,* 1991, Goodrich *et al.,* 1993, Winston *et al.,* 1993, Prentice *et al.,*1994). In our study all allograft recipients received prophylactic ACV. In this study, GCV was not used for prophylaxis, and in the majority of cases, neither was it used pre-emptively on the basis of PCR results. The observation that viral load was significantly higher in patients treated with GCV can be explained by the fact that patients were normally given GCV therapy based on clinical assessment of symptoms rather than on the basis of positive PCR results. Thus, treatment based on PCR positivity was not established as routine practice until the latter part of the study period and does not appear to have significantly influenced the correlation between viral load and disease in this group of patients. Therefore this group represents a unique cohort in which the natural history of CMV in BMT recipients can be assessed. Patients with symptoms of CMV infection were more likely to have been PCR positive for some time prior to the development of symptoms and, as shown in Figure 4.4, peak virus load was correspondingly higher in patients with a higher frequency of PCR positive blood samples.

In order to investigate whether critical viral load thresholds exist above which disease is highly likely we used the multivariate logistic regression model to compute a disease probability - viral load curve. This curve showed that at viral loads below 3.5 $\log_{10}$ genomes/ml blood the disease probability was less than 10% whereas between 3.5 and 4.5 $\log_{10}$ genomes/ml the probability of disease rose 6-fold and reached a plateau of 80% at 5.0 $\log_{10}$ genomes/ml blood. In other transplant patients populations, e.g., liver transplant recipients and AIDS patients, the threshold viral loads for disease are higher than in the BMT recipient (5.5 $\log_{10}$ and 5.0 $\log_{10}$ genomes/ml, respectively). It is likely that virus replication in the BMT patient does not reach particularly high levels, but these patients may be more susceptible to the pathologic effects of CMV viraemia at relatively low levels. It is important to note that in the BMT patient the development of CMV pneumonitis is a particularly serious problem. Early seeding of the lung tissues in the absence of a reconstituted immune system may contribute to disease, (or a reconstituted immune system which may be CMV naïve initially), so that a delayed onset of disease becomes apparent post-engraftment (between 15 and 60 days post transplant) when CMV specific lymphocytes are recruited to the area.

In conclusion, the data in this study demonstrate the central role of viral load in the pathogenesis of CMV in the bone marrow transplant recipient and provides the mechanism by which the previously identified risk factor of recipient seropositivity is associated with disease. These data also provides a rational approach to reducing viral loads below critical levels though antiviral chemotherapeutic targeting of patients most at risk of symptomatic infection, as well as monitoring the success of such interventions. Thus, prophylaxis may work by keeping virus load below a critical level and could explain why a drug such as ACV, with a low in vitro potency against CMV could have a greater than expected effect on CMV disease.

Chapter 5

Studies to investigate the relative contribution of viral dynamics, A-GvHD, T-cell depletion and CMV strain variation to disease in bone marrow recipients

5.0 Introduction

The clinical use of bone marrow transplantation is one of the major success stories from the past 30 years. There are, however, many problems associated with BMT that remain unresolved. The contribution of CMV load in the blood and the risk of disease associated with the reactivation of host virus in the absence of a competent immune system has been discussed in the previous chapter. In addition to the direct effects of CMV viraemia discussed in Chapter 4, there are a number of other factors which may either predispose the BMT recipient to disease, or exacerbate the immunopathology associated with CMV infection.

CMV pneumonia occurs most commonly in patients undergoing allogeneic transplantation for leukaemia or other malignancy. In a study by Meyers *et al.,* (1990) a major risk factor for CMV pneumonia after allogeneic transplant was identified as the occurrence of acute graft versus host disease (A-GvHD). Using univariate analysis, the authors found a significant association between A-GvHD and CMV infection and with CMV disease. In particular, A-GvHD was found to be predictive for CMV pneumonia, though not for gastrointestinal disease. An association between CMV infection and chronic GvHD has also been reported (Lönnqvist *et al.,* 1984). The biological basis of GvHD occurring in the context of HLA matched transplant is still poorly understood, and it is thought that infections with CMV and fungal infections may precipitate rejection of host tissue by the implanted graft. A-GvHD occurs in approximately 50% of HLA matched transplants, probably due to the incompatibility of minor histocompatibility antigens outside the HLA complex on chromosome 6. There are over 37 genes identified in the HLA region, though not all are predicted to be directly related to histocompatibility. Thus, it is still not possible to reliably predict the occurrence of GvHD in the matched unrelated donor setting. Neither is it known which genes are important for graft rejection and A-GvHD. There is some debate as to whether HLA-DP disparities cause GvHD (Nikaein *et al.,* 1994). HLA-C may also be implicated in graft rejection. It is thought that CD4+ helper T-cells may be primarily responsible for the

immunopathogenesis of skin manifestations during A-GvHD. A-GvHD may be explained by the cytokine storm hypothesis that proposes three sequential stages: increased expression of HLA and leukocyte adhesion molecules on target cells, recognition of these antigens by donor T cells which then secrete interleukin-2, and finally activation by IL-2 of other donor cells that then secrete noxious cytokines, notably interleukin-1, tumour necrosis factor (TNF) and interferon gamma (IFN-γ) (Robinson *et al.,* 1994). It is also likely that there is a mixed chimerism of T lymphocytes in the skin during A-GvHD, and the peptide of the HLA groove or a superantigen associated with HLA molecules may act as a stimulatory antigen. CMV antigens have been shown to stimulate some of the skin infiltrating T lymphocytes (Nikaein *et al.,*1994).

A-GvHD may occur at any time during the first 100 days post-transplant, although it occurs most commonly in the early post-graft period. It usually involves the skin and may involve the liver and gastrointestinal tract. It presents as an acute inflammation of tissue and may result in the desquamation of the skin, severe hepatitis and necrosis of the intestinal epithelium in the worst instance. Treatment for A-GvHD includes the use of immunosuppressive agents such as cyclosporin A, methylprednisolone and methotrexate. In some patients, specific depletion of T cells by anti-thymocyte globulin (ATG) is also used. A consequence of this is that host defence against infection is suppressed immunologically and by neutrophil dysfunction, both systemically and in the lung (Cunningham, 1992). Significantly, alveolar macrophages have been found to have impaired chemotaxis, phagocytosis and killing of bacteria and fungi in patients more than 50 days post-transplant (Winston *et al.,* 1982).

The widespread use of allogeneic BMT is limited by the availability of suitable donors. The use of unrelated donor marrow is limited by the development of severe GvHD and resulting graft failure. Therefore, in order to reduce the incidence of GvHD, T-cell depletion was employed for the majority of the allograft recipients in this study. Campath 1 is a rat anti-human (CDw52) monoclonal antibody, which is used routinely ex vivo for T-cell depletion of allogeneic bone marrow and peripheral blood stem cells. In addition, Campath 1 is administered in vivo to achieve an increased

immunosuppression during pre-transplant conditioning as prophylaxis against graft rejection following T-cell depleted BMT. The range of T-cell depletion in the patient group studied here was between 0% to 100%, depending on the underlying malignancy. A disadvantage of T-cell depletion is that the Graft versus Leukaemia effect (GVL) is reduced. The GVL effect is an immunological phenomenon that is important for the control of minimal residual disease (MRD), and reinducing remission post-BMT. It is thought that large granular lymphocytes (LGLs) and natural killer cells (NK) play a role in MRD. In a study by Condiotti *et al.,* (1996), Campath 1 was found to significantly inhibit the binding of LGLs to tumour cells and NK cell cytotoxicity, a factor which may influence the recurrence of malignant disease and subsequent survival of the patient. On the other hand, highly alloreactive T cells also influence the development of disease and survival in BMT patients. Analysis of the frequency of host-reactive helper T lymphocyte precursors (HTLp) and/ or cytolytic T lymphocytes (CTLp) in recipients of matched unrelated (MUD) or HLA-mismatched bone marrow indicated an increased frequency of severe A-GvHD and chronic GvHD with high levels of CTLps and HTLps (Keever-Taylor *et al.,* 1997). Thus, not only the T-cell dose, but also the alloreactive potential of the infused cells can influence the development of A-GvHD and survival in allograft recipients.

It may be that the possession of particular HLA phenotypes predisposes patients to increased CMV infection, disease or death. In a study by Schrier *et al.,* (1994), among HIV patients who developed CMV retinitis, a significant proportion had at least one of three HLA alleles or combinations. In this patient group A2B44, B51 and DR7 were found frequently in patients with CMV retinitis, with a relative risk of 23.8 compared with patients without these alleles. In solid organ transplant patients (heart, kidney and lung), patients possessing the HLA-B7 allele were found to be especially at risk for developing active CMV infection ($p= 0.03$) and CMV disease ($p= 0.03$) (Boland *et al.,* 1993). Active CMV infection in these patients was defined as antigenaemia and/or CMV excretion. In the light of this data we were interested to see if any specific HLA alleles were associated with an increased risk of CMV disease or death in the BMT cohort of patients we had assembled for the viral load studies.

Differences in the biologic behaviour of CMV strains have been described (Brown *et al.*, 1995). It is possible that genetic variation in functionally important genes may influence the virulence of CMV strains. One important component of the CMV virion is the glycoprotein (gB) B protein. The gB protein is the major target for neutralising antibodies (Cranage *et al.*,, 1986), and is also important for virion penetration into cells, transmission of infection from cell to cell and fusion of infected cells (Tugizov *et al.*, 1994, 1995; Navarro *et al..*, 1993). Certain regions of the gB gene are highly variable between different virus strains. There is a highly variable region around the protease cleavage site (between amino acids 406 and 461) which is involved in the fusion processes (Navarro *et al.*, 1993). Based on the nucleotide sequence data for this variable region, a genotyping scheme was proposed (Chou *et al.*, 1992). Strains were assigned one of four gB genotypes, 1 to 4. Studies to examine the association between CMV strain variation and disease have been performed by several groups (Fries *et al.*, 1994; Shepp *et al.*, 1996; Vogelberg *et al.*, 1996; Shen *et al.*, 1996; Rasmussen *et al.*, 1997). In one study, the gB genotype was found to correlate with the clinical outcome in bone marrow transplant patients. In this group, CMV infection with gB type 1 was associated with a lower mortality compared with infection with gB types 2 - 4 (Fries *et al.*, 1994). In another study by Bongarts *et al.*, (1996), gB type 1 was found less frequently in HIV patients with retinitis (17%) than in asymptomatic patients (37%, $p = 0.05$). Types 1 and 2 were found more frequently (31% and 35% respectively) than types 3 and 4 (9%). In renal transplant recipients gB types 1, 2 and 3 were found to be evenly distributed (Vogelberg *et al.*, 1996). In BMT patients the predominant gB type was 1, (54%), with type 2 at 10%, type 3 at 21%, and type 4 at 6% (Fries *et al.*, 1994). The reason for these differences is not clear. It is possible that gB types differ in their cell tropism and site of latency, or that reactivation of virus is different for different strains. It has also been suggested that the type and severity of the immune defect may influence the prevalence of the gB types. Bongarts *et al.*, (1996) found that in HIV patients the detection of gB types 1 and 2 correlated with dissemination of infection. Type 1 infection tended to be found in patients with CMV detected in one compartment only (urine or blood), whereas other strains were found more frequently in patients with infection detected in two or more compartments ($p = 0.01$). Thus the authors propose that CMV infections with gB

type 1 may be less severe and lead less frequently to retinitis. This situation is similar to that in BMT patients, in whom gB type 1 infection correlated with a more favourable clinical outcome (Fries *et al.,* 1994). Thus, it has been suggested that the gB phenotype might provide a virulence marker for CMV (Bongarts *et al.,* 1996).

The processes involved with CMV infection and disease in the BMT patient are complex.

The aim of studies performed for this chapter was to investigate the contribution of factors other than viral load and recipient:donor serostatus to disease and survival in these patients. Other studies have identified risk factors such as A-GvHD, T-cell depletion and CMV gB strain variation in BMT and other immunocompromised populations. We sought to relate these factors, where possible, to the risk factors for disease that we had already identified in our patient cohort. In addition, a number of studies have sought to examine the dynamics of virus infection in the blood as a potential marker of disease (Bowen *et al.,* 1998, Perleson *et al.,* 1996, Neumann *et al.,* 1998). We therefore attempted to make an assessment of the dynamics of virus infection in the blood of our patient cohort in order to determine the rate of virus clearance in the presence or absence of GCV.

5.2 Methods

5.2.1 Glycoprotein B typing of CMV strains from BMT patients

PCR was used to amplify a segment of the CMV gB gene flanking the gp55 cleavage site according to the method of Shepp *et al.,* (1996). This segment varies in size between 550 and 556 bp depending on the strain, (Cranage *et al.,* 1986) and contains the major region of sequence variability within this gene. DNA extracts from the 49 CMV positive patients from BMT patients used for the study of viral load and longitudinal fluctuations (see Chapter 2) were subjected to further analysis using a nested PCR to amplify a 550 bp sequence from the glycoprotein B gene. The oligonucleotide primers used were as follows:

CB1 5' CTGGGAAGCCTCGGAACG

CB2 5' ACCCATGAAACGCGCGGC

for the 1st round PCR, and then,

CB3 5' ACGTACTATCCGTTCCGA

CB4 5' GGCAATCGGTTTGTTGTA

for the second round, nested PCR.

The sequence of the primers correspond to nucleotides 1200 - 1217, 1765 - 1782, 1215 - 1232, and 1750 - 1767 respectively, of the gB gene (Lee *et al.,* 1992).

The reaction mix was optimised with respect to $MgCl_2$, and primer concentrations, for both rounds of PCR. The reaction mix for the first round of the PCR was optimised as follows:

10µl 10x Amplitaq Gold buffer

4µl $MgCl_2$ (1.5mM)

3µl dNTP mixture (200µM of each)

2µl Primer CB1 (200ng)

2µl Primer CB2 (200ng)

0.5µl AmpliTaq Gold polymerase enzyme

75µl SDW

96.5 µltotal volume per reaction tube

0.5 - 1.0µl of the amplicon from the first round PCR was used as target for the second round or "nested" PCR. The Mg ion and primer concentrations for this PCR were also optimised, and the reaction mixture was prepared as follows:

10µl	10x Amplitaq Gold buffer (Applied Biosystems Inc.
6µl	$MgCl_2$ (2.5 mM)
3µl	dNTP mixture (200ìM of each)
5µl	Primer CB3 (500ng)
5µl	Primer CB4 (500ng)
0.5µl	AmpliTaq polymerase enzyme
78µl	SDW
99.5 µl	total volume per reaction tube

Cycling conditions for the PCR were as follows:

1st round PCR

Stage 1	95°C	15 minutes	1 cycle
Stage 2	95°C	1 minute	
	52°C	1 minute	35 cycles
	70°C	2 minutes	
Stage 3	95°C	1 minute	
	52°C	1 minute	2 cycles
	70°C	10 minutes	

2nd round PCR

Stage 1	95°C	15 minutes	1 cycle
Stage 2	95°C	1 minute	
	52°C	1 minute	40 cycles
	70°C	2 minutes	
Stage 3	95°C	1 minute	
	52°C	1 minute	2 cycles
	70°C	10 minutes	

Amplicons from the nested reaction were visualised as described in Chapter 2.3.3 of this thesis. For the restriction endonuclease digestion analysis 10μl of non-purified PCR product was incubated with 1 unit of Mae III (Boehringer Mannheim, Indianapolis) at 55°C overnight. The resulting DNA fragments were separated on a 4% NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME) gel and visualised by ethidium bromide staining. On the basis of the size of the restriction fragment four individual gB groups (1 - 4) were defined according to the restriction patterns obtained from Shepp *et al.,* (1996). The sizes of the restriction enzyme digested DNA fragments for each gB type are shown in Table 5.1.

Table 5.1 Mae III restriction enzyme digestion pattern for the strain typing of CMV: the numbers indicate the lengths of the DNA fragments in base pairs

CMV Group	DNA Fragment sizes
1	281, 138, 137
2	188, 138, 137, 90
3	362, 188
4	215, 202, 139

5.2.2 Statistical Methods to identify the association between HLA type, T-Cell depletion, acute GvHD, donor-recipient CMV serostatus, and CMV Disease/Death

Associations between categorical variables were tested for significance using the Chi-squared test, or Fisher's exact test where appropriate. (See chapter 2 for brief explanations of these statistical tests). Comparisons of the level of T-cell depletion in different groups were tested using the Wilcoxon test.

Three endpoints were considered for the analysis: the development of CMV disease, any death, and death occurring within 180 days of transplant. For each endpoint, two approaches were taken when analysing the data. First, analyses were carried out which considered simply whether the individual developed the endpoint or not. Factors associated with the endpoints were identified using Chi-squared tests or Fisher's exact test, where appropriate. However, since it is known that the time to development of the endpoint is also of interest, a second approach was taken which incorporated information on this time period. Kaplan-Meier analysis and proportional hazards regression models were used to identify factors associated with a longer or slower time to reach the endpoint. Factors found to be associated with the endpoint were then included in a multivariate proportional hazards model to identify factors, which were independently associated with the endpoint. Each factor was included in the model using a forwards selection method. For the analysis of severe CMV disease, patient follow-up was right-censored at the time of death, if they remained free of CMV disease at this time. For the analysis of survival, the follow-up of patients who remained alive was right-censored at the time of the patient's last contact with the unit (analysis of all deaths) or at 180 days following transplant (analysis of early deaths only).

All survival analyses were carried out using the Statistical System Package (SAS) using the PHREG and LIFETEST procedures. Factors included in the analysis were: possession of HLA haplotypes: (A1,A2,A3,A9,A11,A29,B5,B35,Cw7,DR2,DR3), donor and recipient CMV IgG serostatus, and degree of T-cell depletion. It should be

noted at that this analysis was restricted by the fact that this was a retrospective study on the patients samples we had available from earlier studies. Very large numbers of patients are required for this type of analysis, and this initial work was performed in order to evaluate the possibility of examining HLA associations with disease in a typical clinical cohort of this nature. Statistical power is a measure of the extent to which a study is capable of discerning differences or associations which exist within the population under investigation, and is of critical importance whenever a hypothesis is tested by statistics. Conventionally, studies should reach a power level of 0.8, such that four times out of five a false null hypothesis will be rejected by a study. Statistical power may most easily be increased by increasing the sample size (Fox & Mathers, 1997). Adequate power is essential so that effects that exist are not missed.

Had our study been performed as part of a prospective clinical trial, or for a surveillance programme, power calculations to indicate the numbers of patients necessary to achieve statistical significance would have been required. However, it should be noted that the populations of patients receiving bone marrow transplants world-wide are very heterogeneous, and the numbers overall are still very small. It is unlikely that even with studies spanning several years, there would be sufficient numbers of specific HLA haplotypes to achieve statistical power. Studies with larger numbers of patients have been performed. In a large-scale study for the European Bone Marrow Transplantation (EBMT), where data was pooled from several BMT centres, no statistically significant association was found between any HLA alleles and clinical outcome (Ljungman *et al.,* personal communication).

Using the data available (see Table5.4) the HLA alleles used to perform multivariate analysis on were restricted to A2, B7 and B18. The B7 allele was chosen because it had been shown to be associated with CMV infection and disease in other transplant populations (Boland *et al.,* 1993). The B18 allele was chosen because in the larger analysis shown in Table 5.4 patients possessing this allele showed a significant correlation with survival ($p = 0.02$). A similar, though not significant trend was seen with the A2 allele. It should be noted that though these three alleles were the only ones

chosen to be included in the multivariate analysis, 13 of the most prevalent alleles were used for the initial univariate analysis of correlation with overall survival (Table 5.4).

5.2.2 Calculation of the Half-life of virus in the blood

In order to determine the duration of viraemia, and the possible effect of GCV therapy on the clearance of virus from the blood, the half life of virus in the blood was determined using the equation:

$$\frac{\ln t_0 - \ln t_1}{t} = slope$$

the half life of virus in the blood then is calculated as:

$$\frac{\ln 2}{slope} = t_{1/2}$$

where $\ln$ is the natural log, t_0 is the virus load at the first time point, and t_1 is the virus load at the subsequent time point, and t is the number of days over which the change in virus load was measured. Thus, $t_{1/2}$ is the rate of increase or decrease of the virus in the blood. For patients who had more than one episode of DNAemia, the rate of virus increase and clearance was calculated for each peak, and the means of the doubling times and half-lives were calculated. The rate of virus increase and decrease during the first episode of viraemia was also calculated, as were the rates of virus increase and decrease during treatment with GCV. Statistical analysis was performed to determine correlations between the rate of virus increase and clearance and previously identified risk factors.

5.3 Results

5.3.1 Prevalence of Glycoprotein B variants

15 out of the 49 patients who were CMV positive using the surveillance gB PCR (149bp product) were found to be positive using the nested PCR for the larger gB product for gB strain typing. All samples, whether previously PCR positive or negative, from the patients were tested. The results of the restriction digest analysis showed that glycoprotein B types 1 and 2 were the most prevalent in this group of patients. No patients from whom the specific sequence could be amplified were found to have a type 4 infection and only one patient had a type 3 infection. Examples of typical restriction enzyme digests are shown in Figures 5.1 and 5.2.

Table 5.2 Summary of the Mae III restriction enzyme analysis of the glycoprotein B amplified sequence in CMV positive BMT recipients

CMV glycoprotein B type	Number of Patients (%)	Ratio symptomatic: asymptomatic
1	5 (33)	2:3
2	8 (53)	5:3
3	1 (6)	1:0
4	0 (0)	0

Figure 5.1 Mae III restriction enzyme digestion of PCR products showing typical restriction patterns for different strains of CMV. Lanes 1, 2 and 7 correspond to type 2, lanes 3, 4, 6 and 8 to type 1

Figure 5.2 Mae III restriction enzyme digestion of PCR products showing restriction patterns for CMV gB strains. Lane 1 corresponds to type 3, lane 2 to type 2 and lane 3 to type 1. Lane 4 corresponds to a PCR size marker

5.3.2 Statistical analysis of the relative contribution of multiple risk factors for CMV infection and death

Of the 68 allograft recipients for whom data was available for HLA status, 33 (48%) were PCR positive and were included in the earlier analysis on viral load. 40 (58.8%) patients had donors who were CMV IgG positive. Of the recipients, 47 (69.1%) were CMV IgG positive. Information about T-cell depletion was available for 68 of the patients (median level of depletion: 85.1%, range 0% - 100%), of these patients 49 received a marrow with T depletion of between 1% and 100%. 18 patients received a non-depleted marrow. Information on A-GvHD was available for 46 out of 74 allograft recipients. Of these, 18 had A-GvHD and 28 did not. 10/18 patients with A-GvHD were CMV PCR-positive. Individuals with A-GvHD had lower levels of T-cell depletion (median 70.0% versus 93.5% in those without A-GvHD); this difference was statistically significant ($p = 0.04$, Wilcoxon). There was also a trend ($p= 0.08$) towards an increased risk of disease with A-GvHD. There was, however, no difference in the median maximal viral loads in patients with or without A-GvHD (median loads 3.90 and 3.95 respectively, see Figure 5.3). When univariate logistic regression analysis was performed to determine the contribution to disease by recipient seropositivity, virus load in the blood, and the presence of A-GvHD, only virus load was found to be significantly associated with disease (Table 5.3). There was a trend towards the contribution of recipient seropositivity and A-GvHD to disease ($p = 0.05$ and 0.08, respectively), however, in multivariate logistic regression analysis, any statistical significance of recipient serostatus and A-GvHD were abolished ($p = 0.93$ and 0.11 respectively). Venn diagrams summarising the patient numbers in each of the study groups are shown in Figures 5.4 -7.

A total of 14 allograft recipients developed CMV disease during the study period (20.6%; Kaplan-Meier progression rate of 24.5% by 1500 days post-transplant). There were no significant associations between any factor studied, (other than virus load, see Chapter 4), and the development of CMV disease, either when considering the development of disease in a Chi-Squared analysis, or when incorporating time to development of disease using a proportional hazards model.

A total of 35/110 (31%) patients died over the study period (51.5%, Kaplan-Meier rates of 100% by 1500 days, median 657 days, when all deaths were included, and 29.4% by 180 days when only early deaths were included). There was no association in this data set between the occurrence of A-GvHD and death ($p = 0.92$, Chi squared test), nor between PCR status and death ($p = 1.00$, Chi-squared test). The major HLA types in this group of patients and the association between the specific HLA types and death are shown in Table 5.4. There were no significant associations between any specific HLA type and CMV PCR positivity or disease. However, individuals who possessed HLA type A3 were less likely to be PCR positive than those without type A3 ($p = 0.05$).

Possession of HLA type B18 was associated with an increased risk of death ($p = 0.02$) (Table 5.5) in univariate and multivariate analysis with other HLA types.

Proportional hazards models (Table 5.6) show that donor CMV IgG positivity was associated with a reduced risk of death ($p = 0.04$) in univariate analysis, but its significance was reduced in multivariate analysis ($p = 0.07$). There was also a marginal association between T-cell depletion and death, patients given marrow with any degree of T-cell depletion had a reduced risk of death compared to those who were transplanted with non-T-depleted marrow. In multivariate analysis, both possession of HLA type B18 and donor CMV IgG status were independently associated with survival, although in the case of the donor serostatus, this relationship was marginally non-significant. T-cell depletion did not remain significant in multivariate analysis.

When considering only those deaths which occurred within the first 180 days post-transplant, univariate results indicated that possession of HLA types A2 (RH = 2.11, $p = 0.10$) and B18 (RH = 3.01, $p = 0.02$) were associated with an increased risk of death. Possession of HLA type B7 was associated with a slightly reduced risk of death (RH = 0.18, $p = 0.10$). In multivariate analyses, only the possession of HLA type B18 remained independently associated with decreased survival (RH = 2.40, $p = 0.04$).

Table 5.3 Univariate relationships between recipient serostatus, acute graft versus host disease, virus load and CMV disease

Parameter	Odds Ratio	95% CI	p -value
R+D-	6.60	0.95 - 15.72	0.05
Acute GvHD	3.17	0.87 - 11.55	0.08
Virus load (per 0.25 log increase)	1.43	1.12 - 1.82	0.004

Figure 5.4 Summary of CMV PCR positivity in the recipients of allografts or autografts

Figure 5.5 HLA typing data was available for a total of 68/74 patients who received allografts

Figure 5.6 Relationship between allograft and autograft recipients, and their CMV PCR status and availability of A-GvHD data

Figure 5.7 Relationship between CMV PCR status, T-cell depletion and A-GvHD in recipients of allograft

Figure 5.8 A-GvHD in patients with CMV DNAemia: No significant difference in peak virus load was observed between patients who had A-GvHD and those who did not

Table 5.4 Summary of the predominant HLA types of patients and correlation with overall survival

HLA type	Number (%) of patients with HLA type	Number(%) of patients dead	Number (%) of patients alive	p value
A1	23 (34.9)	11 (31.4)	12 (38.7)	0.72
A2	26 (39.4)	16 (45.7)	10 (32.3)	0.39
A3	14 (21.2)	7 (20.0)	7 (22.6)	1.00
A9	8 (12.2)	3 (8.6)	5 (16.1)	0.46
A11	12 (18.2)	5 (14.3)	7 (22.6)	0.58
A29	9 (13.7)	5 (14.3)	4 (12.9)	1.00
B5	8 (12.1)	2 (5.7)	6 (19.4)	0.13
B7	13 (19.7)	7 (20.0)	6 (19.4)	1.00
B18	10 (15.2)	9 (25.7)	1 (3.2)	0.02
B35	8 (12.1)	4 (11.4)	4 (12.9)	1.00
CW7	12 (18.2)	7 (20.0)	5 (16.1)	0.93
DR2	11 (16.7)	6 (17.1)	5 (16.1)	1.00
DR3	20 (30.3)	11 (31.4)	9 (29.0)	1.00

Table 5.5 Relative hazards, 95% confidence intervals and p-values of factors associated with death obtained from proportional hazards analysis

Univariate analyses			
HLA type	**Relative hazard**	**95% CI**	**p-value**
A2	2.11	0.88 - 5.10	0.10
B7	0.18	0.02 - 1.35	0.10
B18	3.01	1.15 - 7.85	0.02

Table 5.6 Relative Hazards, 95% confidence intervals (CI) and p-values of factors associated with death obtained from proportional hazards analysis

Univariate analyses				Multivariate analyses		
Factor	**Relative hazard**	**95% CI**	**p-value**	**Relative hazard**	**95% CI**	**p-value**
B18	2.78	1.25 - 6.20	0.01	2.40	1.06 - 5.43	0.04
donor IgG	0.48	0.24 - 0.97	0.04	0.52	0.26 - 1.06	0.07
T-cell depletion (>0%)	0.53	0.26 - 1.10	0.09	ND	ND	ND

ND = analysis not done

5.3.4 Viral dynamics and control of infection

When each episode of viremia was plotted against time (for reference see the longitudinal profiles of individual patients in Chapter 4 of this thesis), there was a significant difference between the rate of increase of virus and the rate of virus clearance in all patients, i.e. virus levels decreased at a faster rate than they had increased in the blood during any episode of viraemia ($p = 0.0013$, Spearman's Rho = 0.57). When the rate of virus increase during the first episode of viraemia was compared with its corresponding rate of decline, there was a significantly faster rate of virus clearance ($p = 0.005$). The rate of virus clearance was compared between patients who were CMV IgG seropositive and received seropositive marrow (R+D+), combined with patients who were CMV seronegative and received a seropositive marrow (R-D+), i.e. patients who were likely to be protected by either adoptively transferred immunity, and patients who were at risk of reactivating their CMV in the absence of a primed immune system (R+D-). There was no significant difference in the mean ascending ($p = 0.345$, Mann Whitney test) or the mean descending ($p = 1.00$) viral half-life between patients who received a seropositive marrow and patients who were seropositive prior to transplant and were given marrow from a seronegative donor. The half-lives (mean values) of virus in the blood of patients with different R/D serostatus combinations are summarised in Table 5.7. Patients who were seronegative and received a marrow from a seronegative donor i.e. those who experienced a primary infection, were not included in this analysis. The half-life of CMV decay in the blood during GCV therapy was calculated, assuming an exponential decline of virus, and the results subjected to statistical analysis. Nine of the 19 patients who were treated with GCV received the drug during an episode of viraemia, only these patients were included in the analysis. There was no significant difference in the mean rate of virus decline between patients who were treated with GCV and those who were not ($p = 0.33$, Mann Whitney). Neither was there any significant difference in the rate of virus decline between patients who experienced CMV disease and those who did not ($p = 0.27$).

Table 5.7 Correlation of GCV therapy, CMV serostatus and the viral dynamics of CMV in the blood of Bone Marrow Transplant Recipients

Patient ID	CMV serostatus*	GCV therapy	CMV disease	Mean Half-life (days)	
1	1	Y	YES	A 3.93	D 1.83
2	1	Y	YES	A 0.4	D 1.8
3	1	N	YES	A 2.31	D N/A
4	1	N	YES	A 8.88	D 1.68
5	1	N	NO	A 1.32	D 1.73
6	1	Y	NO	A 1.55	D 1.36
7	1	N	YES	A 1.35	D 1.57
8	1	N	YES	A 0.38	D 0.56
9	1	Y	NO	A 13.1	D 3.93
10	1	Y	NO	A 4.7	D 3.92
11	1	N	YES	A 1.47	D 1.36
12	1	N	NO	A 4.33	D 3.22
13	1	N	NO	A 1.32	D 1.11
14	1	N	NO	A 2.15	D 1.31
15	1	N	NO	A 3.44	D 2.74
16	2	N	NO	A 1.84	D 4.29
17	2	N	YES	A 2.89	D 1.41
18	2	Y	YES	A 2.25	D 3.24
19	2	Y	YES	A 1.08	D 1.24
20	2	N	YES	A 1.37	D 1.59
21	2	Y	YES	A 0.84	D 0.96
22	2	N	YES	A 1.30	D 1.27
23	2	N	NO	A 4.7	D 1.61
24	2	N	NO	A 10.22	D 3.83
25	2	Y	NO	A 1.1	D 1.1

* 1 = D+R+ or D+R- 2 = R+D-
Y = YES N = NO A = ascending D = descending

5.4 Discussion

Three risk factors for CMV disease additional to those of viral load and recipient seropositivity in the BMT recipient were assessed in this study: the occurrence of A-GvHD, the role of T-cell depletion and CMV strain variation. In addition, the dynamics of virus replication in the blood and subsequent clearance under the influence of either GCV therapy or the patients' recovering immune system were investigated.

Because differences among gB strains have been reported to influence clinical outcome in a variety of immunocompromised patients, I investigated the prevalence of the four gB genotypes of CMV in blood samples from the bone marrow recipients previously studied (Chapter 4). Unlike other groups, which have examined strain variation in a variety of body compartments, we were limited in this study to DNA extracted from whole blood. Previous studies have generally amplified DNA for genotyping from virus isolated in cell culture. In our laboratory, routine cell culture for CMV was not performed for blood samples from BMT patients because culture methods have generally been superseded by PCR diagnosis, therefore isolates were not available for this retrospective analysis. One advantage of amplifying virus directly from the sample extract is that we were not selecting out virus that had adapted to cell culture. However, in spite of nesting the PCR very few of the samples previously identified as CMV positive were found to be positive for the larger gB product. Out of 49 patients who had a positive PCR result at any time, samples from only fifteen patients could be amplified for gB strain typing, despite repeated attempts at optimising the PCR for greater sensitivity and using a nested PCR approach. These DNA extracts had been repeatedly frozen and thawed and had probably deteriorated significantly. For future work aliquoting the extracts into smaller volumes and avoiding repeated freeze-thawing would be recommended. Of the 15 patients from whose samples the gB fragment could be amplified, 5 (33%) were infected with type 1; 8 (53%) type 2; 1 (6%) type 4, and 0 with type 3. Consistent with other reports, the majority of the patients who had CMV disease had evidence of a type 2 infection. Studies in HIV infected patients have also revealed an increased prevalence of gB

type 2 (Rasmussen *et al.,* 1997). Although mixed infections with two or more strains of CMV have been reported in some organ transplant patients, I found that in the blood of the BMT recipients only one strain was repeatedly amplified and identified by restriction enzyme analysis. This may be because only one body compartment was examined (blood) and other strains may have existed in other sites of infection. It is not clear if it is strain variation alone, or in combination with other factors that influence the virulence of CMV strains. The gB protein is important for viral transmission from cell to cell, and is highly conserved among the herpesviruses (Pereira *et al.,* 1994). It has been shown that a single base change in the gB protein of HSV1 can confer increased neuroinvasiveness in mice (Yuhasz *et al.,* 1993). There is recent evidence that CMV gB types differ in their tropism for T-cells in *in vivo* (Meyer-König *et al.,* 1995). The gB genotype of CMV is also known to be linked to the genotype of another membrane protein, gH. To date, variation in the gH gene has not been shown to be correlated with clinical outcome (Fries *et al.,*1994). Rasmussen *et al.,* (1997) postulate that humoral immunity, which is important for preventing primary infection (and this would include BMT patients reactivating virus in the absence of a primed marrow), is genotype specific, and is unable to neutralise the gB2 strain as well as other strains. The number of patients with gB typing data available was too small for valid statistical analysis, further study in this and other organ transplant populations are required. This data may be of significance since the CMV gB subunit vaccine being developed uses the Towne strain which is known to be gB type 1.

For HIV-infected patients a gB vaccine with all 4 genotypes may be useful for reducing the incidence of infection with multiple strains and the risk of CMV retinitis. Indeed, patients with type 1 infection have been shown to have a reduced risk of retinitis (Bongarts *et al.,* 1996). It is important to stress that isolates with the same gB genotype are not necessarily identical because genetic variations may occur elsewhere in the viral genome which could also affect tissue tropism and pathology. Efforts to define the genetic regions of CMV strains that are associated with virus pathology may provide important information that will help to explain the wide range of consequences in the immunocompromised host.

There is little data available on the association between the HLA haplotype of the BMT recipient, and CMV infection or disease, or for survival post-BMT. In this study a significant association with increased death was noted in patients with the haplotype B18 ($p = 0.02$). When the cut-off date for death was set at 180 days post-transplant, i.e. early death, HLA types A2 and B18 were linked with an increased risk of death, whilst possession of B7 was associated with a reduced risk of death. No other HLA type represented in our analysis was found to have any significant association with CMV infection, disease or death, and only HLA type B18 remained statistically significant in multivariate analysis. Two factors need to be taken into account with this analysis; first, the numbers of patients possessing each haplotype are very small; secondly, the association between specific haplotypes and particular malignant conditions in this patient population is not clear. It may be that some haplotypes are more frequently associated with especially malignant conditions which may influence the success of the bone marrow transplant. It may also be that, since HLA antigens are found in linkage disequilibrium, certain combinations of HLA antigens predispose the patient to increased or decreased survival. Unfortunately, numbers in this study were too small to use linkage disequilibrium to analyse risk for disease or death. Given the enormously heterogenous population undergoing bone marrow transplantation throughout the world, and the fact that many haplotypes exist in linkage disequilibrium, it would be almost impossible to recruit sufficient numbers of patients to achieve statistical power for this type of analysis. A much larger study to determine the association of HLA haplotypes and CMV disease in the European Bone Marrow Transplant cohort has similarly failed to show any statistically significant associations (Ljungman, personal communication).

Of all the patients who experienced CMV infection, 10 experienced A-GvHD. As expected, patients with higher levels of T-cells in the transplanted marrow experienced more A-GvHD than patients who received marrow which was T-cell depleted to any extent. Marrow was T-cell depleted to varying extents depending on the patient's clinical history and the risk of A-GvHD or leukaemia relapse. Purging the donor marrow of T-cells is a very effective means of reducing the risk of A-

GvHD, but the risks of viral infections, tumour relapse and graft rejection are subsequently increased. One solution is to provide BMT recipients post-transplant with T-cells which are responsive to foreign antigens but unresponsive to host HLA antigens. A method described by Rencher *et al.,* (1996), involves the culture of donor T-cells with host derived B lymphoblastoid cells, which serve to activate the T-cells that respond to the host HLA antigens. These activated cells are then removed using fluorescence activated cell sorting (FACS), leaving T-cell populations with helper and cytotoxic T cell responses against foreign antigens, but which are specifically unresponsive to host antigens. Thus the patient benefits from T cell immunity and avoids the risk of developing A-GvHD.

Although the association between CMV disease and the occurrence of A-GvHD was not statistically significant, there was a trend ($p = 0.08$) for patients with A-GvHD to have more CMV disease. When less stringent definitions for CMV disease were used (see Chapter 4.3.1, Plotkin scoring system), this association did reach statistical significance. However, there was no difference between the median viral loads between patients who developed A-GvHD and those who did not. It is possible that the timing post-transplant of the first episode of viraemia is more important for the development of A-GvHD than the peak viral load (Couriel *et al.,* 1996). Again, as expected, donor IgG seropositivity was favourably associated with survival. There was also a marginal association between T-cell depletion and death, when the analysis was performed comparing patients who were transplanted with marrow with any T-cell depletion versus those who were given non-T -depleted marrow.

The results of the study of viral dynamics in these patients indicated a significantly faster rate of virus decline when compared with the rate of increase ($p = 0.0013$). When all patients were considered together, the mean rate of increase of virus was 2.42 days, whereas the rate of decline in the same patients was 1.89 days. Several patients experienced more than one episode of viraemia, and this figure therefore represents the mean of all the peaks for each patient. However, because it is possible that the first episode of viraemia may follow a different pattern under the influence of a severely suppressed immune response (and possibly an unprimed immune system)

the rate of increase and decrease for the first peaks were also considered. The mean ascending rate of virus during an initial episode was 2.41 days and the mean rate of decline was 1.79 days ($p = 0.005$, Spearmans test). There was no significant difference between the rate of increase or clearance of virus when the mean rates of first episode and all episodes of viraemia were compared. It is possible that any advantage conferred by a recovering immune system might be balanced by any immunosuppressive therapy for A-GvHD or graft rejection by the host. Interestingly, virus clearance rates were not significantly different between patients treated with GCV during an episode of viraemia, and those with a natural resolution of viraemia (mean rate of decline = 2.42 days in treated patients verse 1.78 days in non-treated patients, $p = 0.072$, Mann-Whitney). There was, however, a trend for longer clearance times in patients treated with GCV ($p = 0.072$). This might be explained by the myelosuppression caused by the GCV treatment. Patients who remained asymptomatic did not have significantly faster rate of clearance of virus than patients with CMV disease ($p = 0.27$, Mann-Whitney). This type of analysis does have a number of limitations and viral dynamics data should be interpreted with caution. First, it assumes a steady state or pseudo-steady state of virus replication resulting in an exponential increase or decrease of virus in the blood. More frequent sampling of the blood may refine these estimates and perhaps reveal a biphasic increase or decline rate, with an initial sharp change followed by a plateau phase produced by immune modulation or a lack of sufficient target cells to sustain_replication. An investigation of the viral dynamics in both plasma and leucocytes may also provide a more accurate estimate of the rate of virus replication and decline (Perelson *et al.,* 1996). Because the bone marrow transplant recipients are such a heterogeneous group, it may be that a more individualised approach to the investigation of virus dynamics is required to obtain a clearer picture of the roles antiviral therapy and the host's immune response to CMV infection. This type of analysis has been performed for HIV and hepatitis C infections by Perelson *et al.,* 1996 and by Neumann *et al.,* 1998. Five patients in this study experienced breakthrough of virus during GCV treatment - investigation of potential antiviral resistance may reveal the point mutations seen in HIV-positive patients with CMV infection (Bowen *et al.,* 1997).

In conclusion, no other risk factors except virus load and recipient seropositivity was found to be significantly associated with CMV disease in the BMT recipient. T-cell depletion of donor marrow offered survival benefit probably by reducing the risk of A-GvHD, although the advantages of T-depletion are counterbalanced by increased risk of relapse through loss of GVL effect. Although there was a trend for an increased risk of CMV disease in patients with A-GvHD, this did not appear to be related to the peak virus load. CMV strain variation may have contributed to increased disease risk in this patient population, but the number of patients for whom this data was possible was too small for statistical analysis. Finally, no association was found between any HLA type and CMV disease, although possession of B18 was linked with an increased risk of death.

Any association with disease in the BMT patient should be made with caution since CMV pneumonitis, the most frequent manifestation of CMV disease, is thought to owe its aetiology to the early infection of the alveoli rather than to the level of virus in the blood (Couriel *et al.,* 1996).

Chapter 6

Phenotypic and Genotypic analysis of Aciclovir resistant HSV isolates from Immunocompromised Patients

6.1 Introduction

Recurrences of HSV infection in the immunocompetent host are normally self-limiting, but in the immunocompromised patient untreated infections can become chronic and progressive (Seigal *et al.,* 1981; Whitley *et al.,* 1984). Herpes simplex virus (HSV) continues to cause serious disease in immunocompromised patients, such as transplant recipients and those with AIDS. Antiviral therapy with ACV remains the "gold standard" of treatment and suppression of infection in both the normal and immunocompromised host (O'Brien *et al.,* 1989, Wade *et al.,* 1984), but its utility has been limited by the incidence of drug resistance (Erlich *et al.*, 1989, Englund *et al.,* 1990, Safrin *et al.,* 1996). Clinically significant resistance has been seen almost exclusively in the immunocompromised population, where it occurs in up to 5% of patients (Burns *et al.,* 1982; Crumpacker *et al.,* 1982, Englund *et al.,* 1990, Erlich *et al.,* 1989, Wade *et al.,* 1983). ACV-resistant isolates have been identified in immunocompetent patients, although drug resistance has not generally correlated with clinical disease (Lehrman *et al.,* 1986). In fact, work using animal models has shown attenuated virulence of certain ACV-resistant HSV isolates, with reduced mucocutaneous pathogenicity and decreased recurrence of latent disease (Field *et al.,* 1978, Field *et al.,* 1980). Recently, however, there has been a report of chronic ulceration due to ACV resistant HSV in an immunocompetent patient with normal HSV- specific proliferative and CD8 cytotoxic T cell responses and normal NK function (Swetter *et al.,* 1998). Recurrent ACV-resistant genital herpes has also been reported in another immunocompetent patient treated with ACV (Kost *et al.,* 1993). How ACV resistant HSV can evade drug therapy and cause disease is not well understood (Horsburgh *et al.,* 1998).

Most cases of clinical ACV-resistance in HSV are associated with defects in the viral thymidine kinase (TK). In order to inhibit virus DNA replication ACV must be activated by phosphorylation to its triphosphate form. However, unlike the activation of drugs like idoxuridine, it is the virally encoded thymidine kinase (TK) that is responsible for the initial conversion to ACV-monophosphate. Subsequent

phosphorylation occurs under the control of cellular kinases. This specific activation also results in a selective uptake of ACV into infected cells, whereas no significant conversion to ACV monophosphate occurs in uninfected cells. ACV triphosphate has a higher affinity for viral DNA polymerase than the natural substrate deoxyguanosine triphosphate and so it successfully competes for the enzyme, resulting in preferential incorporation into the replicating DNA chain. Chain termination then occurs because the analogue lacks a 3'-hydroxyl group capable of accepting the incoming nucleotide. Additionally, the incorporated ACV cannot be excised by host exonucleases. The polymerase becomes irreversibly bound to the ACV terminated DNA, resulting in an inactivated enzyme complex. Thus, by this selective mechanism of action the drug activity is confined to viruses that encode a specific TK able to recognise ACV as a substrate. A second consequence of this selectivity is that resistance to the drug may arise by alteration of either of the virus-specific functions involved in the process, thymidine kinase or DNA polymerase. Because the sensitivity of HSV to ACV is due largely to the viral TK, mutations in the HSV TK or viral DNA polymerase can confer resistance to the drug (Collins *et al.,* 1991, Birch *et al.,* 1990, Sacks *et al.,* 1990). Deficiencies in the virus TK accounts for the overwhelming majority of instances of resistance (Hill *et al.,* 1991). Resistant viruses may either fail to produce TK or produce it at very low levels. These viruses are termed TK deficient (TK^D) variants and ACV resistance is defined by an inability to activate the drug. Although ACV resistance is generally due to a total lack of functional viral TK, occasionally resistance can be the result of an alteration to the TK protein, so that the normally wide spectrum of TK activity excludes ACV (Darby and Field, 1981, Ellis *et al.,* 1987, Hill *et al.,* 1991, Kost *et al.,* 1993). HSV strains deficient in the ability to induce a functional TK appear spontaneously and have been recognised for many years. The thymidine kinase polypeptides expressed are truncated as a result of insertions or deletions giving rise to frameshifts which result in premature stop signals and a truncated polypeptide (Summers *et al.,* 1975, Kit *et al.,* 1987).

Table 6.1 gives a summary of the type of genotype changes in the viral TK that lead to specific mutant phenotypes. In addition to the mutations that result in a complete elimination of enzyme function, mutations can occur which alter the substrate binding

properties of thymidine kinase. Such variants are known as altered thymidine kinase mutants (TK ^{alt}). HSV TK is composed of two identical sub-units, each containing 377 amino acids. The native protein has binding sites for both the natural nucleoside substrates and the phosphate donor ATP. Based on predictive amino acid sequences and the recently determined three-dimensional structure it is thought that there is an ATP binding site at the N terminus between residues 49 and 66 (Collins and Darby, 1991, Brown *et al.,* 1995).

Table 6.1 Mutations in the thymidine kinase and DNA polymerase genes of HSV which result in a loss of sensitivity to ACV

Mutant type	Genetic lesion	Outcome
TK negative	Nucleotide substitution	Amino acid substitution or premature termination
	Nucleotide addition	Frame shift normally resulting in premature termination
	Nucleotide deletion	Frame shift, normally resulting in premature termination
TK altered	Nucleotide substitution	Amino acid substitution
DNA polymerase	Nucleotide substitution	Amino acid substitution

Nucleotide sequence analysis has shown that in TK altered mutants it is single base substitutions that are responsible for the phenotype changes. Darby *et al.,* (1986), have reported mutations in two TK altered variants which lead to predicted amino acid substitutions at amino acid residues 168 and 176 (substitution of alanine by threonine at residue 168 in mutant B3 and arginine by glutamine at residue 176 in mutant TR7). Both mutants retained their substrate binding capacity for ATP, but were resistant to ACV. These changes occurred within a region of high homology between HSV 1 and HSV 2, at residues 161 - 193, suggesting that this region may be responsible for nucleoside binding. Table 6.2 summarises the base substitutions, base deletions and insertions that have been reported by various groups (Darby *et al.,* 1996, Kit *et al.,* 1994, Horsburg *et al.,* 1998, Sasadeusz *et al.,* 1997, Gaudreau *et al.,* 1998). Sasadeusz *et al.,* (1997) have reported "hotspots" of mutations within the TK gene that are responsible for phenotypic resistance to ACV. These take the form of single

G deletions or insertions in a string of seven Gs between nucleotides 434 and 439, and C insertions or deletions also occur commonly in a string of five Cs further downstream between nucleotides 884 and 887 (Harris *et al.,* personal communication).

Viruses may also be resistant to DNA polymerase inhibitors such as phosphonoacetic acid and phosphonoformic acid (PFA). These viruses are cross-resistant to ACV. Since the mechanism of action of phosphonoacetic acid depends entirely on the inhibition of the viral DNA polymerase, it has been concluded that mutations in the polymerase gene are likely to explain the cross-resistance to ACV. DNA polymerase mutants isolated in cell culture may be subdivided according to their sensitivity to the pyrophosphate analogue, phosphonoacetic acid, as compared to ACV. Some mutants are resistant to both compounds, whereas others are resistant to ACV but sensitive or hypersensitive to phosphonoacetic acid. Sequence studies of the polymerase gene revealed single base substitutions giving rise to amino acid changes. Because it is essential to retain the replicative function of the enzyme, changes in the DNA polymerase must be conservative and in regions that influence function. Larder *et al.,* (1987), have proposed seven function domains (I - VII) within the HSV polymerase gene. Mutants with changes in region III were resistant to ACV, but remained sensitive to phosphonoacetic acid, suggesting that this region may be involved with nucleoside binding rather than with pyrophosphate exchange. Conversely, mutations in region II generally conferred resistance to both ACV and phosphonoacetic acid, leading to the conclusion that this region is the pyrophosphate-binding site of the enzyme. Pyrophosphate analogue resistant mutants arise readily, perhaps because they are less effective than ACV against the DNA polymerase, thus allowing more rounds of replication to occur before inhibition and therefore allowing more opportunity for mutations to appear (Larder *et al.,* 1987).

Genetic analysis of DNA polymerase mutants indicates that resistance is due to subtle mutations involving putative functional regions of the enzyme. These changes do not result in an alteration of the ability of the virus to replicate in culture. Thus, as with TK altered mutants, the pathogenesis of these variants would not be expected to

change. Polymerase mutants have been shown to replicate normally at peripheral sites, to establish latency and to reactivate in the same way as wild type virus. There is some evidence, however, to suggest that the replication of these viruses in the central nervous system is impaired, and cutaneous infection in mice with a number of polymerase mutants showed a higher survival rate than infection in mice with the parental strain (Larder *et al.,* 1985). Attenuation was also observed with clinical isolates introduced at peripheral sites and inoculated into the brain. It should be noted that all of this work was carried out in immunocompetent animals. The pathogenesis in immunosuppressed animals and humans is likely to be different, for example, TK deficient mutants in nude mice can induce severe skin lesions.

The incidence DNA polymerase mutants in clinical isolates is extremely rare, but thymidine kinase minus and TK altered mutants are frequently seen. It is known that the TK of HSV is non-essential for virus replication in actively growing cells in vitro. However, failure to express the enzyme in vivo (e.g. in animal models) results in a significant reduction in neurovirulence (Field *et al.,* 1978; Tenser *et al.,* 1981). TK minus mutants grow less well at peripheral sites and fail to reactivate from latent infections of mouse sensory ganglia because of the TK mutation (Coen *et al.,* 1989, 1994, Jacobson *et al.,* 1993). They are also less neurovirulent when injected directly into the brain. Although Tenser and colleagues (1981) were unable to establish either an acute or latent ganglionic infection in mice following ocular injection, other workers (Efstathiou *et al.,* 1989) have managed to achieve colonisation of mouse ganglia using a deletion mutant of HSV 1. Latency was established to a degree, but the virus was unable to reactivate. Likewise, a genetically engineered TK mutant was unable to establish latency in mice, but latency was achieved in rabbits (Meignier *et al.,* 1988).

TK altered mutants that maintain function in all respects other than ACV recognition would be expected to behave like wild type virus in vivo in terms of their virulence and pathogenesis. Darby *et al.,* (1981) showed that strain S1, an altered TK mutant, had pathogenesis at the peripheral site that was identical to the wild type virus. Moreover, latency was established and virus was subsequently reactivated.

Table 6.2 Phenotypic and Genotypic analysis of HSV 1 & 2 TK genes from clinical isolates and plaque purified strains

Patient isolate/strain	TK phenotype (%TK activity compared with TK+ control)	TK Genotype	
		Nucleotides	Amino acids
A1⑤	TK-	Added G, 439.5	Stop 82aa downstream
B4I	TK low/altered	A → C, 391	Thre → Pro 131
D6I	TK low/altered	G → A, 1007	Cys → Try 336
E7	TK-	*	271, 272, 273 aa
F11i	TK low/altered	Deleted C, 553	Stop 225 aa downstream
F12	TK-	Deleted G, 439	Stop 37 aa downstream
H15	TK-	C → T, 188	Thre → Ile, 63
I16 i	TK low/altered	C → T, 646	Arg → Cys, 216
I17 i	TK low/altered	A → C, 186	Lys → Asn, 62
J18	Not known	C → T, 841	Arg → stop, 281
K20i	TK low/altered	C → T, 664	Arg → Cys, 222
L21i	TK low/altered	Added G x 2, 436.5	Stop 37 aa downstream
N23	TK-	C → T, 860	Thr → Met, 287
O24	TK-	Deleted C, 464	Stop 27 aa downstream
Q27	TK low/altered	Mixed G & A, 1007	Mix Cys & Tyr, 336
R28	TK low/altered	Added G, 436.5	Stop 160 aa downstream
S29	TK low/altered	C → A, 170	Pro → His, 57
T30	TK low/altered	Deleted C, 669	Stop 185 aa downstream
U32	TK-	A → C, 601	Thr → Pro, 201
V33	TK-	Deleted A, 187	Stop 23 aa downstream
W34	TK low/altered	G → A, 527	Arg → Gln, 176
X35	TK-	A → T, 312, & T → C, 890	Gln → His, 104, & Leu → Ser, 297
B3①	TK altered	Not stated	Ala →Thr, 168
TR7	TK altered	Not stated	Arg→Glu, 176
S	TK altered	Not stated	Cys→Tyr, 336
1②	TK altered	Not stated	His→Tyr, 223
GG3 - 5③	TK low/altered	Not stated	Val→Met, 187
1737-14④	TK -	+GG within G homopolymer	Premature termination, 28kDa product
89-063-1 89-353-1	TK- TK-	-C within C homopolymer +C within C homopolymer	Truncated product of 29kDa Truncated product of 32kD
15923⑥	100% TK -	+G within G homopolymer	Truncated product of 29kDa
14849	90% - 100% TK-	+G within G homopolymer	Truncated product of 29 kDa
14848	90% - 100% TK	-C within C homopolymer	Truncated product of 26kDa
14847	100% TK -	-C within C homopolymer	Truncated product of 26kDa
15583	100% TK -	+G within G homopolymer	Truncated product of 29 kDa
15582	100% TK -	-C within C homopolymer	Truncated product of 26kDa

* = Substitutions found: CGC (Arg)→GTC (Val), aa 271,
CCC (Pro) →AGC (Ser), 272, GAC (Asp) → CGG (Arg), aa 273

① Darby *et al.*,, 1996
② Kit *et al.*,, 1987
③ Horsburg *et al.*,, 1998
④ Sasadeusz *et al.*,, 1997
⑤ Gaudreau *et al.*,, 1998
⑥ Swetter *et al.*,, 1998

HSV disease in the immunocompromised host is distinct from that seen in normal patients as the likelihood of disseminated infection is increased in the absence of a fully functioning immune system. Thus, these patients require extended periods of therapy or prophylaxis and the virus is exposed to the drug for longer. In common with the situation in cell culture, the resistant viruses isolated from these patients are predominantly TK deficient. This may be due to the fact that wild type clinical isolates of HSV contain a small proportion (1/1000 - 1/10000) of TK deficient variants which are selected and enriched from the population in the presence of ACV. The majority of resistant variants isolated from clinical samples have a TK deficient phenotype, although TK altered/low producer mutants have been isolated (Hill *et al.,* 1991). HSV mutants that are severely impaired for TK activity and therefore ACV resistant in vitro have been reported to express low levels of TK that are sufficient for certain phenotypes to reactivate. Hwang *et al.,* (1994), have reported the characterisation of an isolate that encodes a gene predicted to express an inactive-truncated TK polypeptide due to a single base insertion mutation. Despite this mutation, and resulting frameshift, the virus was found to retain low levels of full-length TK polypeptide. Although the isolation of ACV resistant viruses from immunocompetant individuals is still relatively rare, there have been several reports of patients who have suffered severe HSV disease despite ACV therapy. These patients have shed resistant viruses that were reported to have no detectable TK activity and/or full length TK polypeptides (Ljungman *et al.,* 1990, Safrin *et al.,* 1991). Whether or not TK activity can be dissociated from neurovirulence has been debated. Chatis and Crumpacker (1991) and Erlich *et al.,* (1989), have reported a plaque-purified TK^D isolate from an AIDS patient that, due to a single base substitution, produced a full length protein and retained full neurovirulence. It may be, however, that the assay used to detect TK activity was of low sensitivity and failed to detect a TK low-producer phenotype. The detection of DNA polymerase mutants from clinical isolates is very rare.

Wade *et al.,* (1982) have reported the recovery of ACV resistant virus from 2% of BMT recipients receiving a single course of ACV therapy. A second course of therapy yielded 7% resistant isolates. It is possible that in patients such as the BMT

patients described in chapters 4 and 5 who receive ACV prophylaxis for substantial periods of time, the recovery of resistant viruses may be higher. A similar concern applies to patients with AIDS who are also subjected to ACV prophylaxis or therapy over long periods. In AIDS patients episodes of acute HSV infection can result in HIV transcription and subsequent increases in plasma virus load (Mole *et al.,* 1995). It is unclear whether opportunistic infections in HIV patients contribute directly to clinical progression of HIV disease through T-cell activation and subsequent HIV replication, or through T-cell destruction, or both. Reactive HSV infections are found frequently in HIV infected patients, and in vitro and in vivo studies suggest that HSV 1 can activate latent HIV or enhance replication of the virus (Gendelman *et al.,* 1986, Golden *et al.,* 1992, Mosca *et al.,* 1987, Heng *et al.,* 1994). It is likely, however, that the overall impact on the patient may not be very great since the increase in mRNA is only transient, and short-term increases have been associated with other infections.

The aim of this chapter was to investigate the phenotypic changes of HSV isolates obtained through routine surveillance of immunocompromised patients on ACV therapy or prophylaxis. Functional studies for TK and DNA polymerase activities were performed at the Wellcome Laboratories at Beckenham, Kent. Viruses that were found to be of a TK deficient or altered phenotype were subjected to sequence analysis. Although phenotyping studies were performed for DNA polymerase mutants, no genotyping was done with respect to this gene.

6.2 Patients

Three HIV positive patients and one patient undergoing BMT had lesions from which resistant viruses were isolated. One resistant isolate was obtained from each of three HIV positive patients, and six samples from the patient undergoing bone marrow transplantation for Chronic Granulocytic Lymphoma (PH+) who had received a T-cell depleted allograft. This patient experienced recurrent oral lesions from which HSV was isolated, in spite of receiving prophylactic ACV. The patient had no PCR positive results for CMV during the surveillance period of 34 days and died of acute respiratory distress 49 days after transplant. Limited clinical details for the three HIV positive patients were available because the files had been archived. However, all three patients were on AZT anti-retroviral therapy and experienced multiple ano-genital lesions from which HSV was isolated during the surveillance period.

6.3 Methods: Phenotypic Analysis

6.3.1 Culture of Aciclovir Resistant Herpes simplex Mutants

Swabs from oral and ano-genital lesions were taken in viral transport medium (Hank's Lactalbumin, bovine serum albumin Factor V, penicillin, amphotericin B, buffered with 7.5% sodium bicarbonate) and inoculated onto Human Embryo Lung (HEL) fibroblast cell monolayers. The cells were grown with Eagles Medium supplemented with 10% foetal calf serum, 1% glutamine and 2% penicillin. 0.1 ml of the inoculum was mixed with 1ml of culture medium and inoculated onto the cell monolayers and incubated at 37°C for one hour before the medium was refreshed and the cultures re-incubated for 1 to 7 days. If cytopathic effect typical of HSV was observed during the assessment period the culture fluid was harvested, clarified by centrifugation at 1000 x g for 5 minutes and aliquoted into 1ml volumes. The aliquots were stored at –20°C for further testing.

6.3.2 Titration of Virus Stocks

The method used to titrate the potency of the virus stock was that of Reed and Meunch (1938). A 50% tissue culture infectious dose ($TCID_{50}$) is that concentration of inoculum that will infect 50% of the cultures to which it is added. 0.2 ml of serially diluted virus stock taken from the aliquots described in Section 6.3.1 (from 10^{-1} to 10^{-8}) was added to confluent HEL cell monolayers grown in glass tissue culture tubes. Four such tubes containing 1.8 ml of maintenance medium, (Eagles Minimum Essential Medium, MEM, with 5% foetal calf serum, FCS, 1% glutamine and 2% penicillin and gentamicin) were inoculated with each dilution of virus. The tubes were incubated at 37°C for one hour and then the culture medium was replaced with fresh maintenance medium. The cultures were incubated at 37°C and examined for cytopathic effect (CPE) daily.

6.3.3 Calculation of 50% Endpoint for Virus Titration

Once 50 -70% CPE was observed, (around 5 - 7 days post inoculation), the endpoints were calculated as follows:

Virus dilution	Number of Tubes		Accumulated values		Accumulated mortality ratio	Accumulated mortality ratio %
	Degenerate	healthy	Degenerate	Healthy		
10^{-1}	4	0	14	0	14/14	100
10^{-2}	4	0	10	0	10/10	100
10^{-3}	4	0	6	0	6/6	100
10^{-4}	2	2	2	2	2/4	50
10^{-5}	0	4	0	6	0/6	0
10^{-6}	0	4	0	10	0/10	0

Accumulated values for infected tubes were calculated by adding the total number of infected tubes from highest dilution to the lowest. Accumulated values for healthy tubes were calculated by adding the total number of healthy tubes from the lowest dilution to the highest.

From these figures the 50% endpoint was calculated as follows: First, the proportionate distance (PD) of the 50% endpoint was calculated.

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

$$= \frac{100 - 50}{100 - 0} = \frac{50}{100} = 0.5$$

this figure was multiplied by the negative log of the dilution factor

$$0.5 \times (-1) = -0.5$$

The corrected PD was then added to the negative log of the dilution next above the 50% mortality to give the 50% endpoint.

$$(-0.5 + (-5)) = -5.5$$

Thus the 50% endpoint was calculated as $10^{-5.5}$

6.3.4 Sensitivity Testing for Aciclovir

A 1 mM stock solution of ACV was made in maintenance medium and diluted to give concentrations of 1, 50 and 100 μM. Aliquots of titrated virus were thawed and diluted to 1/200 x TCID50. Two tubes of confluent HEL cells for each concentration of ACV, plus extra tubes for ACV free controls were used for each virus to be tested. The virus to be tested was added to the two tubes and incubated at 37°C for an hour before the medium was removed and replaced with fresh medium containing the appropriate concentration of ACV. The tubes were incubated at 37°C for 48 hours and examined for CPE. The following known ACV sensitive and resistant viruses obtained from Dr Graham Darby at Wellcome laboratories were set up in the same way as controls:

Sc16 Sensitive parent strain

TP2.5 DNA polymerase mutant

S1C TK altered mutant

S6a TK minus mutant

Viruses were recorded as sensitive if they grew at ACV concentrations between 0.5 and 5μM and resistant if they grew at concentrations of 15 to 40μM.

Assays for the measurement of HSV thymidine kinase and DNA polymerase activity described below were performed at the Wellcome Research Laboratories, Beckenham, Kent.

6.3.5 HSV Thymidine Kinase Assay

This method is a modification of the method described by Klemperer *et al.,* (1967). The enzyme extract was prepared as follows:

24 well Linbro plates were seeded with BHK (baby hamster kidney, TK-) cells at a density of 10^5 cells per well. The wells were infected with virus at an MOI (multiplicity of infection) of 5 – 10, and incubated overnight at 37°C. The medium was then removed and the cells were washed with PBS and drained, before the addition of 100μl of Tris buffer (0.01M, pH 7.4). The plates were frozen at –20°C, thawed and the cells scraped off into the Tris buffer before ultrasonicating to release virus from the cells. 25 μl aliquots were used for the enzyme assay.

The enzyme assay was then performed on each virus extract.

Reaction mixture:

25 μl 25mM $MgCl_2$

25 μl 25 mM ATP

25 μl 150 μM [14C] thymidine

25 μl 0.1M phosphate buffer

25 μl extract

The reaction was kept on ice throughout.

The reaction mixture was incubated at 37°C for 0, 5, 10 and 15 minutes, and each reaction was stopped by plunging the tube in a boiling waterbath for 2 minutes, and then rapid chilling on ice. 50 µl of each reaction was spotted onto DE81 paper (Whatman) discs which were then washed three times with 1mM formic acid. The discs were air-dried and transferred to tubes containing 2ml of scintillant and counted using a scintillation counter.

6.3.6 Isolation of HSV induced DNA Polymerase

The following reagents were used for this assay:

TBS (Tris buffered saline)

0.5M Tris pH 7.4

0.15M NaCl

Buffer A

0.05M Tris pH 7.8
0.001M $MgCl_2$
0.001M DTT (dithiothreitol)

Buffer B

0.02M Tris pH 7.8
0.1M NaCl
0.015M $MgCl_2$
0.008M DTT

Buffer D

0.05M Tris pH 7.8
0.001M EDTA
0.001MDTT
5% Glycerol

Wintersberger A

0.02M Tris-HCl pH 7.6

1mM EDTA
0.5mM DTT
10% glycerol

All solutions were autoclaved before use and the DTT was added immediately prior to use.

6 litres of HeLa S-3 cells were grown at a seeding density of 5 –8 x 10^5 cells per ml. The cells were pelleted by centrifugation and resuspended in 1000 – 1500 ml of Joklik's modified MEM (Sigma) + 10% newborn calf serum. The cells were infected with HSV at an MOI of 10. After 1 hour 2 litres of medium was added and the cells were incubated for 14 – 16 hours.

The cells were then pelleted by centrifugation and washed twice in TBS. The pellet was resuspended in 30ml of Buffer A. 0.06ml of Triton X-100 was added, vortexed and the nuclei were pelleted by centrifugation for 15 mins at 2300 rpm using a Sorvall ss-34 rotor. The supernatant was poured off (cytoplasmic fraction) and the nuclei were resuspended in 10ml of Buffer B. To this 3ml of 4M NaCl was added, and the mix was incubated at 4°C for 30 minutes. The nuclear and cytoplasmic fractions were centrifuged separately at 10 000 x g for two hours. The supernatants were then dialysed against Buffer D overnight.

The products were then clarified by centrifugation at 3000g for 20 minutes, and the nuclear and cytoplasmic extracts combined. The entire sample was applied to 40ml (2.5cm x 8cm) DEAE columns previously equilibrated with Buffer D. The columns were washed with 20ml of Buffer D, and a gradient of 40ml of Buffer D + 0.1M NaCl and 40ml Buffer D + 0.7M NaCl was applied. All the fractions thus obtained were assayed for polymerase activity.

6.3.7 DNA polymerase Assay

Assay Mixture:
1 part 500mM Tris pH 8.5

1 part 1mM dATP

1 part 1mM dCTP

1 part 1mM dGTP

1 part 1mM [3H] dTTP (~ 50cpm/pmol)

0.3 part 400 mM $MgCl_2$

0.0117 part 1M DTT

for the assay:

26.5 µl of the assay mixture

11 µl of DNA (activated)

12.5 µl enzyme

the mixture was incubated for 30 minutes, then 20 µl spotted onto 1.7cm square of DE-81 paper (Whatman). The squares were dried and washed four times for 5 minutes each with 5% Na_2HPO_4, then once in distilled water and twice with 95% ethanol. The squares were then dried and counted in scintillant.

6.3.8 HSV Plaque Reduction Assay for ACV Susceptibility Testing

24 well Linbro plates were seeded with Vero cells at a density of 2 x 105 cells/ml/well. The cells were grown to confluency, the growth medium was decanted off, and the monolayer was infected with HSV in a volume of 200 µl per well. The cultures were adsorbed for 1 hour at 37°C in 5% CO_2, and the infected cultures were overlayed with 1.5 ml/well of maintenance medium prepared in carboxymethyl cellulose (CMC) containing doubling dilutions of ACV. The plates were incubated for 48 – 72 hours depending on the plaque size at 37°C in 5% CO_2.

After incubation the cultures were fixed with 10% formalin in PBS for 1 hour, and stained with Crystal violet (0.5% in 20% methanol).

The plaques were counted using an inverted microscope and expressed as a percentage of the uninfected controls to determine the 50% inhibitory concentration.

Typical expected IC50 values in Vero cells are as follows:

HSV type I virus: 0.1 – 1.5 µM ACV

HSV type II virus 0.3 – 3.0 μM ACV.

6.3.9 CMC overlay recipe

Glasgows Modified MEM	10%
New born Calf serum	2%
Sodium bicarbonate	2%
Penicillin/streptomycin	2%
Glutamine	1%

Prepared in a 1% solution of CMC.

6.4 Genotypic analysis of ACV Resistant HSV Isolates

6.4.1 Hirt Extraction of HSV DNA

The HSV isolates were grown in a 25cm^2 tissue culture flask of confluent human embryo lung fibroblasts until 80% CPE was observed (approximately 1 -3 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 minutes. 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°C overnight to precipitate chromosomal DNA. Chromosomal DNA was pelleted by microcentrifugation at 13,000 x g for 30 minutes and the supernatant containing genomic DNA treated with 100µg Proteinase K for at least 2 hours at 72°C. Viral DNA was subsequently extracted by adding an equal volume of phenol-chloroform, vortexing and pulse centrifugation at 13,000g. The top layer was transferred to another microtube and the phenol-chloroform extraction process repeated once again. 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 DNA pellet was washed with 70% ethanol and 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.

6.4.2 Primers for the Amplification of HSV TK Sequences

A set of four primers to amplify sequences from the thymidine kinase gene of HSV type 1, strain SC16 were synthesised by R&D Systems, Oxford, UK, as shown in Figure 6.1a.

TK1A: 5' d-GCG CCT TCT AGA AGC GCG TAT G

TK1B: 5' d-GAA GGG CCC CCA ACA CGA TGT T

TK1C: 5' d-TCA TCT TCG ACC GCC ATC CCA T

TK1D: 5' d-TAT TGT CTC CTT CCG TGT TTC A

All primers were synthesised 5' phosphorylated and column purified.
Amplification of the HSV DNA performed using primers TK1A and B to produce an amplicon 564 bp in length, and primers TK1 C and D produced an amplicon 674 bp in length.

6.4.3 Optimisation of the PCR to amplify HSV TK DNA

Initially, the PCR was optimised to amplify products from Hirt extracted DNA, but in order to avoid passaging the virus through cell culture, the PCR was re-optimised so that culture fluid (CF) taken from tubes in which virus had grown through the highest concentration of ACV could be used directly as target. 2µl of the CF was added to a PCR mix containing 10x Optiperform buffer, (20mM Tris-HCl, pH8.0, 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Nonidet P-40, 0.5% Tween-20, Bioline, UK), 2.5 mM MgCl, 200µM dNTP mix, 4 units of Bio-X-Act Taq polymerase enzyme and 300ng each of primers TK1A and TK1B spanning the region from 497 bp to 1119 bp of the TK gene, and TK1C and TK1D spanning the region from 1012 bp to 1665 bp.

For each pair of oligonucleotide primers the PCR was 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. For the primer set AB 1.5 mM $MgCl_2$ was found to be optimal, and for the primer set CD, 2.5 mM $MgCl_2$ was optimal. The optimal primer concentration was determined by varying the concentration of oligonucleotide primers from 50ng to 400ng in each reaction. 200ng each of primers A and B, and 300ng of primers C and D were found to be optimal.

97μl of the reaction mix was aliquoted into 0.5ml eppendorf tubes and overlayed with mineral oil (Sigma).

The *Taq* polymerase and primer concentrations were re-adjusted to optimise amplification, and the volume of target was reduced to 2μl, in order to counteract possible inhibitory effects on the PCR caused by residual ACV in the culture fluid. It was found that by increasing the amount of enzyme and primer and reducing the amount of target to 2μl it was possible to dilute out the inhibitory effects of ACV without affecting the sensitivity of the assay. Thermal cycling conditions were also optimised to obtain the most sensitive annealing temperature (ranging from 37°C to 55°C) for both primer pairs. The thermal cycling conditions for each set of primers were optimised as follows:

Primers A - B

Stage 1	95°C	5 mins	1 cycle
Stage 2	95°C	1 min	
	60°C	1 min	38 cycles
	72°C	1 min	

Primers C - D

Stage 1	95°C	6 mins	1 cycle
Stage 2	95°C	1 min, 15 secs.	
	58°C	2 min	40 cycles
	70°C	2 min	
Stage 3	95°C	1 min, 15 secs.	
	58°C	2 min	2 cycles
	70°C	10 min	

Figure 6.1 The DNA sequence of the TK gene of HSV-1 is shown. Two reference strain sequences of HSV1 (CL101, and SC16) and one HSV2 (333) strain are shown in order to indicate the level of natural sequence variation between strains and types. Regions of sequence variation between the strains are shown in red. The primer sequences AB and CD are highlighted and show that there is very little sequence homology with the HSV-2 gene at the primer binding sites.

Figure 6.2 The predicted amino acid sequence of the TK gene is shown for the two reference strains of HSV-1 (CL101 and SC16) and one HSV-2 strain (333). The full-length protein is predicted to be of 377 amino acid residues for HSV-1 and 376 amino acids for HSV-2. Sequence homology is indicated by a red line, and variation is indicated in green.

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                                                  10        20        30        40        50        60        70
                                                  TAGAAGCGCGTATGGCTTCGTACCCCGGCCATCAGCACGCGTCTGCGTTCGACCAGGCTGCGCGTTCTCGCGGC
HSVTKstrain CL101 1.SEQ(1>1799)  ->TAGAAGCGCGTATGGCTTCGTACCCCTGCCATCAACACGCGTCTGCGTTCGACCAGGCTGCGCGTTCTCGCGGC
HSV-2.333 1.SEQ(1>1659)          ->            ATGGCTTCTCACGCCGGCCAACAGCACGCGCCTGCGTTCGGTCAGGCTGCTCGTGCGAGCGGG
SC16 1.SEQ(1>1131)               ->            ATGGCTTCGTACCCCGGCCATCAGCACGCGTCTGCGTTCGACCAGGCTGCGCGTTCTCGCGGC

                                  80        90       100       110       120       130       140       150
                                  GCAACCGACGTACGGCGTTGCGCCCTCGCCGGCAGCAAGAAGCCACGGAAGTCCGCCBGGAGCAGAAAATGCCC
HSVTKstrain CL101 1.SEQ(1>1799)  ->GCAACCGACGTACGGCGTTGCGCCCTCGCCGGCAGCAAGAAGCCACGGAAGTCCGCCTGGAGCAGAAAATGCCC
HSV-2.333 1.SEQ(1>1659)          ->CCGACGGCCGCGCGGCGTCCCGTCCTAGCCATCGCCAGGGGGCCTCCGAAGCCCGCGGGGATCCGGAGCTGCCC
SC16 1.SEQ(1>1131)               ->GCAACCGACGTACGGCGTTGCGCCCTCGCCGGCAGCAAGAAGCCACGGAAGTCCGCCCGGAGCAGAAAATGCCC

                                 160       170       180       190       200       210       220       23
                                 TACTGCGGGTTTATATAGACGGTCCCCACGGGATGGGGAA-A-AMCCACCACCACGCAACTGCTGGTGGCCCTG
HSVTKstrain CL101 1.SEQ(1>1799)  ->TACTGCGGGTTTATATAGACGGTCCTCACGGGATGGGGAA-A-ACC-ACCACCACGCAACTGCTGGTGGCCCTG
HSV-2.333 1.SEQ(1>1659)          ->TGCTGCGGGTTTATATAGACGGACCCCACGGGGTGGGGAAGACCACCACCTCCGCGCAGCTGATGGAGGCCCTG
SC16 1.SEQ(1>1131)               ->TACTGCGGGTTTATATAGACGGTCCCCACGGGATGGGGAA-A-A-CCACCACCACGCAACTGCTGGTGGCCCTG

                                    240       250       260       270       280       290       300
                                    CGCGCGACGATATCGTCTACGTACCCGAGCCGATGACTTACTGGCAGGTGCTGGGGGCTTCCGAGACAATCGCG
HSVTKstrain CL101 1.SEQ(1>1799)  ->CGCGCGACGATATCGTCTACGTACCCGAGCCGATGACTTACTGGCAGGTGCTGGGGGCTTCCGAGACAATCGCG
HSV-2.333 1.SEQ(1>1659)          ->CGCGCGACAATATCGTCTACGTCCCCGAGCCGATGACTTACTGGCAGGTGCTGGGGGCCTCCGAGACCCTGACG
SC16 1.SEQ(1>1131)               ->CGCGCGACGATATCGTCTACGTACCCGAGCCGATGACTTACTGGCGGGTGCTGGGGGCTTCCGAGACAATCGCG

                                       320       330       340       350       360       370       380
                                       TCTACACCACACAACACCGCCTCGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGACAAGCGCC
HSVTKstrain CL101 1.SEQ(1>1799)  ->TCTACACCACACAACACCGCCTCGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGACAAGCGCC
HSV-2.333 1.SEQ(1>1659)          ->TCTACAACACGCAGCACCGTCTGGACCGCGGCGAGATATCGGCCGGGGAGGCGGCGGTGGTAATGACCAGCGCC
SC16 1.SEQ(1>1131)               ->TCTACACCACACAACACCGCCTCGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGACAAGCGCC

                                          400       410       420       430       440       450       460
                                          TAACAATGGGCATGCCTTATGCCGTGACCGACGCCGTTCTGGCTCCTCATATCGGGGGGGAGGCTGGGAGCTCA
HSVTKstrain CL101 1.SEQ(1>1799)  ->TAACAATGGGCATGCCTTATGCCGTGACCGACGCCGTTCTGGCTCCTCATGTCGGGGGGGAGGCTGGGAGTTCA
HSV-2.333 1.SEQ(1>1659)          ->TAACAATGAGCACGCCTTATGCGGCGACGGACGCCGTTTTGGCTCCTCATATCGGGGGGGAGGCTGTGGGCCCG
SC16 1.SEQ(1>1131)               ->TAACAATGGGCATGCCTTATGCCGTGACCGACGCCGTTCTGGCTCCTCATATCGGGGGGGAGGCTGGGAGCTCA
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                                          470       480       490       500       510       520       530       540
                                          CCCCGCCCCCGGCCCTCACCCTCATCTTCGACCGCCATCCCATCGCCGCCCTCCTGTGCTACCCGGCCGCGCGAT
HSVTKstrain CL101 1.SEQ(1>1799)  ->      CCCCGCCCCCGGCCCTCACCCTCATCTTCGACCGCCATCCCATCGCCGCCCTCCTGTGCTACCCGGCCGCGCGAT
HSV-2.333 1.SEQ(1>1659)          ->      CCCCGCCCCCGGCCCTCACCCTTGTTTTCGACCGGCACCCTATCGCCTCCCTGCTGTGCTACCCGGCCGCGCGGT
SC16 1.SEQ(1>1131)               ->      CCCCGCCCCCGGCCCTCACCCTCATCTTCGACCGCCATCCCATCGCCGCCCTCCTGTGCTACCCGGCCGCGCGAT

                                          550       560       570       580       590       600       610       620
                                          TTATGGGCAGCATGACCCCCCAGGCCGTGCTGGCGTTCGTGGCCCTCATCCCGCCGACCTTGCCCGGCACAAACA
HSVTKstrain CL101 1.SEQ(1>1799)  ->      TTATGGGCAGCATGACCCCCCAGGCCGTGCTGGCGTTCGTGGCCCTCATCCCGCCGACCTTGCCCGGCACAAACA
HSV-2.333 1.SEQ(1>1659)          ->      TCATGGGAAGCATGACCCCCCAGGCCGTGTTGGCGTTCGTGGCCCTCATGCCCCCGACCGCGCCCGGCACGAACC
SC16 1.SEQ(1>1131)               ->      TTATGGGCAGCATGACCCCCCAGGCCGTGCTGGCGTTCGTGGCCCTCATCCCGCCGACCTTGCCCGGCACAAACA

                                          630       640       650       660       670       680       690       7
                                          TGTTGGGGGCCCTTCCGGAGGACAGACACATCGACCGCCTGGCCAAACGCCAGCGCCCCGGCGAGCGGCTTGACC
HSVTKstrain CL101 1.SEQ(1>1799)  ->      TGTTGGGGGCCCTTCCGGAGGACAGACACATCGACCGCCTGGCCAAACGCCAGCGCCCCGGCGAGCGGCTTGACC
HSV-2.333 1.SEQ(1>1659)          ->      TCCTGGGTGTCCTTCCGGAGGCCGAACACGCCGACCGCCTGGCCAGACGCCAACGCCCGGGCGAGCGGCTTGACC
SC16 1.SEQ(1>1131)               ->      TGTTGGGGGCCCTTCCGGAGGACAGACACATCGACCGCCTGGCCAAACGCCAGCGCCCCGGCGAGCGGCTTGACC

                                          710       720       730       740       750       760       770
                                          CTATGCTGGCCGCGATTCGCCGCGTTTACGGGCTGCTTGCCAATACGGTGCGGTATCTGCAGGGCGGCGGGTCGT
HSVTKstrain CL101 1.SEQ(1>1799)  ->      CTATGCTGGCCGCGATTCGCCGCGTTTACGGGCTGCTTGCCAATACGGTGCGGTATCTGCAGGGCGGCGGGTCGT
HSV-2.333 1.SEQ(1>1659)          ->      CCATGCTGTCCGCCATTCGCCGTGTCTACGATCTACTCGCCAACACGGTGCGGTACCTGCAGCGCGGCGGGAGGT
SC16 1.SEQ(1>1131)               ->      CTATGCTGGCCGCGATTCGCCGCGTTTACGGGCTGCTTGCCAATACGGTGCGGTATCTGCAGGGCGGCGGGTCGT

                                          790       800       810       820       830       840       850
                                          GGGAGGATTGGGGACAGCTTTCGGGGACGGCCGTGCCGCCCCAGGGTGCCGAGCCCCAGAGCAACGCGGGCCCAC
HSVTKstrain CL101 1.SEQ(1>1799)  ->      GGGAGGATTGGGGACAGCTTTCGGGGACGGCCGTGCCGCCCCAGGGTGCCGAGCCCCAGAGCAACGCGGGCCCAC
HSV-2.333 1.SEQ(1>1659)          ->      GGGAGGACTGGGGCCGGCTGACGGGGGTCGCCGCGGCGACCCCGCGCCCCGACCCCGAGGACGGCGCGGGGTCTC
SC16 1.SEQ(1>1131)               ->      GGGAGGATTGGGGACAGCTTTCGGGGACGGCCGTGCCGCCCCAGGGTGCCGAGCCCCAGAGCAACGCGGGCCCAC

                                          860       870       880       890       900       910       920       930
                                          CCCATATCGGGGACACGTTATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACGGCGACCTGTACAACC
HSVTKstrain CL101 1.SEQ(1>1799)  ->      CCCATATCGGGGACACGTTATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACGGCGACCTGTATAACC
HSV-2.333 1.SEQ(1>1659)          ->      CCCGCATCGAGGACACGCTGTTTGCCCTGTTCCGCGTTCCCGAGCTGCTGGCCCCCAACGGGGACTTGTACCACA
SC16 1.SEQ(1>1131)               ->      CCCATATCGGGGACACGTTATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACGGCGACCTGTACAACC
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                                          940       950       960       970       980       990      1000      1010
                                          TTGCCTGGGCCTTGGACGTCTTGGCCAAACGCCTCCGTCCCATGCACGTCTTTATCCTGGATTACGACCAATCGC
HSVTKstrain CL101 1.SEQ(1>1799) ->        TTGCCTGGGCCTTGGACGTCTTGGCCAAACGCCTCCGTCCCATGCACGTCTTTATCCTGGATTACGACCAATCGC
HSV-2.333 1.SEQ(1>1659)         ->        TTGCCTGGGTCTTGGACGTCTTGGCCGACCGCCTCCTTCCGATGCATCTATTTGTCCTGGATTACGATCAGTCGC
SC16 1.SEQ(1>1131)              ->        TTGCCTGGGCCTTGGACGTCTTGGCCAAACGCCTCCGTCCCATGCACGTCTTTATCCTGGATTACGACCAATCGC

                                         1020      1030      1040      1050      1060      1070      1080      10
                                          CCGGCTGCCGGGACGCCCTGCTGCAACTTACCTCCGGGATGATCCAGACCCACGTCACCACCCCAGGCTCCAT-A
HSVTKstrain CL101 1.SEQ(1>1799) ->        CCGGCTGCCGGGACGCCCTGCTGCAACTTACCTCCGGGATGGTCCAGACCCACGTCACCACCCCAGGCTCCAT-A
HSV-2.333 1.SEQ(1>1659)         ->        TCGGGTGTCGAGACGCCCTGTTGCGCCTCACCGCCGGGATGATCCCAACCCGCGTCACAACCGCCGGGTCCATCG
SC16 1.SEQ(1>1131)              ->        CCGGCTGCCGGGACGCCCTGCTGCAACTTACCTCCGGGATGATCCAGACCCACGTCACCACCCCAGGCTCCAT-A

                                         1100      1110      1120      1130      1140      1150      1160
                                          ACGATCTGCGACCTGGCGCGCACGTTTGCCCGGGAGAT-GGGGGAGGCTAACTGAAACACGGAAGGASMCRATAC
HSVTKstrain CL101 1.SEQ(1>1799) ->        ACGATCTGCGACCTGGCGCGCACGTTTGCCCGGGAGAT-GGGGGAGGCTAACTGAAACACGGAAGGAGACAATAC
HSV-2.333 1.SEQ(1>1659)         ->        A-GATACGCGACCTGGCGCGCACGTTTGCCCGCGAGGTGGGGGAGTTTAGTTCAAACACGGAA-G-CCCGA-A-
SC16 1.SEQ(1>1131)              ->        ACGATCTGCGACCTGGCGCGCACGTTTGCCCGGGAGAT-GGGGGAGGCTAACTGA

                                         1180      1190      1200      1210      1220      1230      1240
                                          AAGGCMWCCCGCGCKATGACGGCAATAAAARRACAGAATAAAASGCAYKGKTGTYGKGTSGTKTGTYCATAARCG
HSVTKstrain CL101 1.SEQ(1>1799) ->        AAGG-AACCCGCGCTATGACGGCAATAAAAAGACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCATAAACG
HSV-2.333 1.SEQ(1>1659)         ->        AAGGCCTCCCG-GCGATGACGGCAATAAAAGAACAGAATAAAAGGCATTGTTGTCGTGTGGTGTGTCCATAAGCG

                                         1250      1260      1270      1280      1290      1300      1310      1320
                                          GGGTTCGGKSCCAGGGCTGGCACYSTRTCRRYACCCCACCGAARAMCSSAKYGGGCCGATCCGACCTTGTTTTCG
HSVTKstrain CL101 1.SEQ(1>1799) ->        GGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCG-AGACCCCATTGGG
HSV-2.333 1.SEQ(1>1659)         ->        GGGTTCGGGGCCAGGGCTGGCACCGTATCAGCACCCCACCGAAAAACGGAGCGGGCCGATCCGACCTTGTTTTCG

                                         1330      1340
                                          CTGTACTCCTTGTGCTTT
HSV-2.333 1.SEQ(1>1659)         ->        CTGTACTCCTTGTGCTTT
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                 MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRXEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWXVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITMGM
                         10        20        30        40        50        60        70        80        90       100       110       120       130
SC16.prot.PRO    MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITMGM 13C
HSV2.333prot.PF  MASHAGQQHAPAFGQAARASGPTDGRAASRPSHRQGASEARGDPELPTLLRVYIDGPHGVGKTTTSAQLMEALGPRDNIVYVPEPMTYWQVLGASETLTNIYNTQHRLDRGEISAGEAAVVMTSAQITMS 13C
CL101TK.prot.PF  MASYPCHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWQVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITMGM 13C

                 PYAVTDAVLAPHXGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWWEDWG
                        140       150       160       170       180       190       200       210       220       230       240       250       260
SC16.prot.PRO    PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWREDWG 26C
HSV2.333prot.PF  TPYAATDAVLAPHIGGEAVGPQAPPPALTLVFDRHPIASLLCYPAARYLMGSMTPQAVLAFVALMPPTAPGTNLVLGVLPEAEHADRLARRQRPGERLDLAMLSAIRRVYDLLANTVRYLQRGGRWREDW 26C
CL101TK.prot.PF  PYAVTDAVLAPHVGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWWEDWG 26C

                 QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMXQTHVTTPGSIPTICDLARTFAREMGEAN-
                        270       280       290       300       310       320       330       340       350       360       370
SC16.prot.PRO    QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.  377
HSV2.333prot.PF  GRLTGVAAATPRPDPEDGAGSLPRIEDTLFALFRVPELLAPNGDLYHIFAWVLDVLADRLLPMHLFVLDYDQSPVGCRDALLRLTAGMIPTRVTTAGSIAEIRDLARTFAREVGGV.  377
CL101TK.prot.PF  QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQTHVTTPGSIPTICDLARTFAREMGEAN.  377
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6.4.4 Purification of the HSV TK Amplicons

HSV TK amplicons were purified from a 1% low melting point agarose gel (Sigma, molecular biology grade) using the Gene Clean II kit (Bio -101, USA) according to the manufacturers' instructions. Briefly, the DNA fragments of interest were 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 slices were 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 10,000x*g* for one minute and the supernatant removed. The pellets were 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). 2μl aliquots of the purified HSV TK DNA fragments were then re-analysed on a 1% agarose gel in comparison to a λ *HindIII/EcoR1* DNA marker.

6.4.5 Direct Dideoxy Sequencing of the HSV TK Gene

The purified HSV DNA was then directly sequenced using the Sanger dideoxy chain termination procedure using dimethylsulphoxide (DMSO) and the Sequenase 7-Sequencing Kit with Sequenase Version 2.0 T7 DNA polymerase (USB). 5μl target DNA (100-200ng) was mixed with 1μl primer (150ng/μl) and 1μl DMSO. The oligonucleotide sequencing primers were the same as the forward primers used for PCR amplification shown in section 6.4.2. The mixture was boiled for 3 min to denature the double stranded DNA, then snap frozen in liquid nitrogen to keep the DNA single stranded and to allow primer annealing. To the primer annealed DNA, 2μl 5x Sequenase reaction buffer (200mM TRIS-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl), 2μl nucleotide labelling mix (1.5μM each of 7-dGTP, dTTP, dCTP) diluted 1 in 5 with SDW, 1μl DTT (0.1M solution), 0.5μl [α-^{35}S] dATP (5μCi, specific activity 1000 Ci/mmol, 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 in a thermal cycler). 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. After 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.

6.4.6 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% bis-acrylamide, BDH), 15ml filtered 10x TBE and 52.5ml SDW were dissolved on a heated stirrer for 1 hour at 50°C. The container was covered to prevent evaporation. After dissolution, the mixture was allowed to cool to room temperature, 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) heat resistant 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 "T", "C", "A" and "G" 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 for 12 - 36 hours. The DNA sequence was read directly from the film over a light box and compared with the HSV TK sequences obtained from the EMBL database. The LaserGene (DNAStar) software was used to analyse the sequence data and align the results from the clinical isolates with the reference strains GG, CL101 and SC16 (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast).

6.4.7 Cloning of HSV TK Sequences into pUC18 plasmid

The primers used to amplify the HSV DNA were pre-phosphorylated and therefore the purified amplicons were suitable for cloning into a pUC 18 vector (Pharmacia, UK) following Klenow filling to permit blunt ended ligation into the plasmid. 2 units of the Klenow fragment of DNA polymerase 1 were added to the PCR amplified 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 HSV 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

Sma1 cut dephosphorylated pUC18 DNA and all ligation reagents but no insert DNA. The ligations were incubated for 24 - 48 hours at 16°C.

6.4.8 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, pH 6.6) and incubated on ice for 20 minutes. The cells were harvested by centrifugation, as before, and the bacterial cell pellet was resuspended in 20ml of 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.

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

400µl of competent JM109 cells was 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 (pre-warmed 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.

6.4.9 Small-scale (Miniprep) Purification of Plasmid DNA

The selected colonies from Section 6.4.9 were inoculated into 5ml of LB containing 50 μg/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 inverted 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 10 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 10 000 x g. 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 10 000 x *g* in a microcentrifuge. The pellets were washed twice with 300μl 70% ethanol and then finally dried in air. The plasmid DNA pellets were resuspended in 200μl of TE (pH 8.0) and 5μl of RNase A (from a 10 mg/ml stock of 10 mg/ml RNase A (Boehringer Mannheim), 10mM Tris-HCl (pH 7.5) (Sigma), and 15mM NaCl) was added to each sample before incubating at 37°C for 30 min. The plasmid DNA was

extracted from the solution with phenol:chloroform, 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, i.e. >14 clones, the Qiagen Minipreps DNA Purification system was used (Qiagen, UK). Selected colonies were grown up overnight in 5ml of LB broth with ampicillin and the cell pellet harvested by centrifugation as described above. The cells were resuspended in 250µl Solution P1 (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100µg/ml RNase A), lysed in 250µl Solution P2 (0.2M NaOH; 1% SDS (w/v)) for 5 min at room temperature and then neutralised by the addition of 500µl P3 (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 to individual wells of strips of columns provided with the miniprep kits and pulled through the matrix using a vacuum manifold. The matrix was washed with 1ml of solution PB and then washed twice with solution PE. The matrix was allowed to dry under vacuum for 1 min and the bases of the columns were blotted before being replaced in the vacuum manifold above collection tubes and the DNA was eluted in 50µl sterile distilled water (SDW) by vacuum pressure for one minute.

In order to confirm the presence of the insert in pUC18, 5µl of the plasmid DNA prepared above was digested with 1 unit each of *Hind III* and *Eco RI* (Boehringer Mannheim) 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.

6.4.10 Plasmid Sequencing of HSV TK

Plasmid DNA from recombinant colonies was extracted using a standard plasmid mini-preparation procedure (section 6.4.10) and 5µg of DNA subsequently used for sequencing. Five clones from each HSV PCR were sequenced, and in order to ensure the fidelity of *Taq* polymerase in the amplification of HSV TK, 5 clones of the laboratory strains SC16, S1C and S6a were also subjected to identical PCR

amplification conditions and sequencing. The sequence of recombinant clones was determined as described in section 6.4.6 using the Sequenase 7-deaza-dGTP Sequencing Kit and the forward and reverse primers universal to the phage M13 regions of the pUC18 vector.

6.4.11 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 15 ml falcon tube and centrifuged at 400 x 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 ml 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.

6.4.12 *Annealing of Single Stranded DNA Templates to the Sequencing*

The denatured DNA solution from Section 6.4.12 (approximately 25μl eluate) was divided into 7μl aliquots, and each aliquot was 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 required.

The labelling and termination reactions were performed as described in Section 6.4.5 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 described in Section 6.4.6.

6.5 Results

6.5.1 Phenotyping of ACV Resistant Isolates

A total of 53 samples and 6 control virus strains were screened for ACV sensitivity of which nine were found to be ACV resistant (Table 6.3).

Three samples, one each from three HIV positive patients, were found to be resistant to ACV in vitro with IC_{50} values of 56.3, 45.6 and 21.4 μM ACV respectively (Table 6.3). Normally sensitive type 1 strains of HSV have an expected IC50 range of 0.1-1.5μM and type 2 strains 0.3-3 μM ACV. All three HSV isolates were from swabs of ano-genital lesions, and serotyping using fluorescein labelled monoclonal antibodies revealed two isolates to be HSV type 2 and one as type 1. All the isolates were thymidine kinase deficient when tested by the thymidine kinase assay (Section 6.3.5), but sensitive to ACV in virus DNA polymerase assays (Section 6.3.6).

A further six isolates from mouth, lip and throat lesions from a bone marrow transplant recipient were also found to be resistant to ACV in vitro. Two of the isolates were found to have thymidine kinase activity but were borderline resistant in the DNA polymerase assay. The earliest isolate from this BMT recipient grew at a 10μM concentration of ACV and was therefore classified as resistant and subjected to enzyme assays. A second isolate, although apparently sensitive to ACV in vitro, grew at higher concentrations of drug than sensitive isolates, and was therefore also subjected to enzyme function analysis. This isolate was also found to be borderline resistant using the DNA polymerase assay (Table 6.3).

Table 6.3 Summary of phenotype analysis of resistant clinical isolates obtained from three HIV patients and one bone marrow transplant recipient. DNA polymerase inhibition measures the IC50 of ACV-triphosphate on viral DNA polymerase activity. Wild type virus (sensitive strain) normally has an IC50 of 0.024μM, whilst a known polymerase mutant has an IC50 of 0.728μM. For the TK assay, sensitive type 1 strains of HSV have an expected IC50 range of 0.1 - 1.5μM and type 2 strains 0.3 - 3 μM ACV

Patient Type	Site of Lesion	HSV Type	IC50 μM ACV	Sensitive/ Resistant	TK	DNA Pol Inhibition	DNA pol Sensitive/Resistant	phenotype
HIV	genital	2	56.3	Resistant	Negative	0.004	Sensitive	TK-
HIV	genital	2	45.6	Resistant	Negative	0.041	Sensitive	TK-
HIV	?	1	21.4	Resistant	Negative	0.037	Sensitive	TK-
BMT	Throat	1	4.8	Sensitive	Positive	0.234	Sens/Resistant	?
	Mouth	1	4.0	Sensitive	Positive	0.254	Sens/Resistant	?
	Mouth	1	42.6	Resistant	Negative	0.036	Sensitive	TK-
	Lip	1	29.9	Resistant	Negative	0.041	Sensitive	TK-
	Mouth	1	35.1	Resistant	Negative	0.036	Sensitive	TK-
	Lip	1	16.12	Resistant	Positive	0.04	Sensitive	TK^{alt}

6.5.2 Genotyping of ACV Resistant Isolates

Sequencing results obtained for the ACV resistant variants were compared with the parent strain SC16 (Darby *et al.*,, 1986) using the LaserGene (DNAStar) software package. The results of the changes in amino acid sequence as compared with two reference strains (CL101, and SC16) for isolates from each patient are shown in Figures 6.2a-d. A summary of the nucleotide and predicted amino acid changes seen in all isolates is given in Table 6.4.

Table 6.4 Genotyping results for HSV isolates from four patients treated with ACV

Patient (No.) & Isolate	TK Activity	Mutation (Nucleotide)	TK Genotype Amino Acid
1a	Negative	G deletion 386 C→T 125 C→T 609 A→G 1042	Stop at aa, 133 Leu→Pro, 42 Isoleu→Isoleu, 203 Leu→Leu, 347
2a	Negative	T→C 85 C→T 171	Leu→Ser, 28 His→Tyr, 57
3a	Negative	T→C 85 G→T 109 C→T 125 C→T 171	Leu→Ser, 28 Ala→Thr, 36 Leu→Pro, 42 His→Tyr ,57
4a	Positive	A→G 1042	Leu→Leu, 347
4b	Positive	C→T 125 A→G 1042	Leu→Pro, 42 Leu→Leu, 347
4c	Negative	C→T 125	Leu→Pro, 42
4d	Negative	C→T 125	Leu→Pro, 42
4e	Negative	C→T 125 C deletion 1032	Leu→Pro, 42 Stop aa, 347
4f	Positive	C→T 125 T→G 1022 A→G 1042 G→T 1115	Leu→Pro, 42 Cys→Gly, 341 Leu→Leu, 347 Met→Isoleu, 372

Table 6.4 summarises the genotype changes seen in the ACV-resistant isolates from patients 1 – 4. The leucine to proline change was seen in Patients 1 and 3 and also in four of the isolates from Patient 4. A comparison with a number of published HSV-1 sequences revealed that this was a common polymorphism at this site. In fact, at amino acid 42 there is a proline in strain SC16 and a leucine in strains GG and CL101 (GG sequence not shown). Since all of these strains are predicted to encode a full-length protein, it is unlikely that this change was responsible for the resistant phenotype seen in isolate 4c.

The changes from leucine to serine at aa 28, alanine to threonine at aa 36, and histidine to tyrosine at aa 57 were not seen in the two reference strains used (Figure 6.2). However, translation of the sequences with these changes suggested a predicted full-length protein (Figure 6.3). This is also true of the changes from cysteine to glycine at aa 341 and methionine to isoleucine at amino acid 372. The A to T base change at nucleotide 1042 was non-coding, and variability at this site was seen in a number of reference strains using the BLAST searches and Lasergene alignments.

The resistant isolate from Patient 1 had a G deletion at nucleotide 386, which resulted in a frameshift and predicted truncated protein sequence (Figure 6.4a). This isolate also had base substitutions at nucleotide 125 (C to T, deduced amino acid change from leucine to proline at aa residue 42), and at nucleotides 609 and 1042 (base changes C to T, and A to G, respectively, both non-coding). An autoradiograph showing the G deletion is shown in Figure 6.5a and the predicted truncated polypeptide sequence (133 amino acids) is shown in Figure 6.2a.

The ACV-resistant isolate 4e was also found to have a single base deletion at nucleotide residue 1032, resulting in a frameshift and a predicted truncated protein of 347 amino acids instead of the full length 377 amino acid product (Figure 6.2d).

The resistant isolates from Patients 1 and 2 were serotyped as type 2 when assayed using the monoclonal antibody typing kit, but sequence analysis of both the cloned DNA and direct sequenced PCR amplicons revealed an HSV-1 sequence. It is possible that these patients carried and reactivated more than one HSV infection and

the isolates that grew through the ACV and were amplified by PCR were the HSV-1 viruses. Although the PCR primer TK1A was essentially identical to the HSV-2 sequence, all of the other PCR primers were significantly different, and would not have also amplified any HSV-2 viruses that may have been present in case of a mixed infection. Serotyping results were not available when the PCR primers and study were designed, and ways of overcoming this discrepancy are discussed later.

Following pages:

Figure 6.3 Alignment maps of the predicted amino acid sequences of HSV TK of 4 patients from whom virus isolates were obtained. Each patient with multiple resistant isolates is shown together with two prototype HSV sequences for comparison. The continuous red bar indicates sequence homology and breaks of different colours highlight changes.

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                        MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANI
                                  10        20        30        40        50        60        70        80        90        10
HSVTKCL101.prot.PR( MASYPCHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWQVLGASETIANI
HSVTKSC16.prot.PRO  MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANI
PATIENT 2a   85 Tto MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANI
PATIENT 2a  171 C ( MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANI

                        YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPG
                                 110       120       130       140       150       160       170       180       190       20
HSVTKCL101.prot.PR( YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHVGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPG
HSVTKSC16.prot.PRO  YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPG
PATIENT 2a   85 Tto YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALPCYPAARYLMGSMTPQAVLAFVALIPPTLPG
PATIENT 2a  171 C ( YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALPCYPAARYLMGSMTPQAVLAFVALIPPTLPG

                        TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAI
                                 210       220       230       240       250       260       270       280       290       30
HSVTKCL101.prot.PR( TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWWEDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAI
HSVTKSC16.prot.PRO  TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAI
PATIENT 2a   85 Tto TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAI
PATIENT 2a  171 C ( TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAI

                        NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN-
                                 310       320       330       340       350       360       370
HSVTKCL101.prot.PR( NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQTHVTTPGSIPTICDLARTFAREMGEAN.
HSVTKSC16.prot.PRO  NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 2a   85 Tto NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 2a  171 C ( NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
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                                 MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA
                                          10        20        30        40        50        60        70        80        90       100       110       120
HSVTKCL101.prot.PRO              MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWQVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA
HSVTKSC16.prot.PRO               MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA
PATIENT 3a  85 Tto C.            MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA
PATIENT 3a  171 C to             MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA
PATIENT 3a  125 C to             MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA
PATIENT 3a  109 G to             MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQESTEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSA

                                 PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALPCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSV
                                          140       150       160       170       180       190       200       210       220       230       240       250
HSVTKCL101.prot.PRO              PYAVTDAVLAPHVGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSV
HSVTKSC16.prot.PRO               PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSV
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PATIENT 3a  171 C to             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALPCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSV
PATIENT 3a  125 C to             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALPCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSV
PATIENT 3a  109 G to             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALPCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSV

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                                          270       280       290       300       310       320       330       340       350       360       370
HSVTKCL101.prot.PRO              QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQTHVTTPGSIPTICDLARTFAREMGEAN.
HSVTKSC16.prot.PRO               QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 3a  85 Tto C.            QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 3a  171 C to             QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
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                                 MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
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HSVTKSC16.prot.PRO               MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
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PATIENT 4c 125 C to              MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
PATIENT 4d 125 C to              MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
PATIENT 4e 125 C to              MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
Patient 4e C deletio             MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
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PATIENT 4f 125 C to              MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
PATIENT 4f 1022 T to             MASYPGHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRPEQKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANIYTTQHRLDQGEISAGDAAVVMTSAQITM
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                                         140       150       160       170       180       190       200       210       220       230       240       250
HSVTKCL101.prot.PRO              PYAVTDAVLAPHVGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWWED
HSVTKSC16.prot.PRO               PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
PATIENT 4a  1042 A t             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
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PATIENT 4d 125 C to              PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
PATIENT 4e 125 C to              PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
Patient 4e C deletio             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
PATIENT 4f 1042 A to             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
PATIENT 4f 125 C to              PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
PATIENT 4f 1022 T to             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED
PATIENT 4f 1115 G to             PYAVTDAVLAPHIGGEAGSSHAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGSWRED

                                 QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN-
                                          270       280       290       300       310       320       330       340       350       360       370
HSVTKCL101.prot.PRO              QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQTHVTTPGSIPTICDLARTFAREMGEAN.
HSVTKSC16.prot.PRO               QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
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PATIENT 4c 125 C to              QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 4d 125 C to              QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 4e 125 C to              QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
Patient 4e C deletio             QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTPG.
PATIENT 4f 1042 A to             QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 4f 125 C to              QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 4f 1022 T to             QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALRQLTSGMIQTHVTTPGSIPTICDLARTFAREMGEAN.
PATIENT 4f 1115 G to             QLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREIGEAN.
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6.6 Discussion

Herpes simplex infections in the immunocompromised host can cause potentially life threatening infections, leading to pneumonia (Liebau *et al.,* 1996), hepatitis (Shanley *et al.,* 1995), retinitis (Cunningham *et al.,* 1996) and severe ulcerative lesions. Resistance to ACV is still rarely seen in the immunocompetent host, but in immunocompromised patients it is increasingly observed where low levels of the drug are used for prophylaxis often over long periods (Englund *et al.,* 1990, Gaudreau *et al.,* 1998). Clinical disease due to resistant virus is usually characterised by a chronic, progressive ulcerative mucocutaneous disease with prolonged shedding of virus. Although transmission of ACV resistant virus has not been reported to date, widespread use of ACV, particularly for the treatment of genital herpes in the immunocompetent population, raises the concern that this may occur in the near future (Blower *et al.,* 1998). Identification of the mechanisms of resistance using phenotypic and genotypic assays may aid the awareness of resistance in the population and may ultimately aid the design of second generation drugs to combat the problems of antiviral resistance.

Recently, isolates have been identified which retain a small but detectable level of TK activity (1% – 5% by enzymatic assays), but test as ACV resistant by susceptibility assays (Hill *et al.,* 1991). These viruses are designated TK-low producer (TK^{lp}) mutants. TK deficient mutants can establish and maintain latency, but are unable to reactivate in the mouse model, suggesting that they should be unable to reactivate in the patient (Efsthaniou *et al.,* 1989, Tenser *et al.,* 1989, Coen *et al.,* 1989). Recently, however, there have been reports of reactivated TK^{D} clinical isolates (Safrin *et al.,* 1991, Sasadeusz *et al.,* 1996). Some of these isolates have been shown to express activity of TK at levels even lower than the TK^{lp} strains. These levels are below the level of detection of conventional assays and have been termed "ultralow", showing that amounts of TK activity required for activation are far lower than first appreciated (Sasadeusz and Sacks, 1996). HSV isolates have also been demonstrated to exhibit high degrees of heterogeneity, which may result in difficulties in characterisation of DNA sequences and protein products (Sacks and Rennie, 1991, Sacks *et al.,* 1989).

It is now known that phenotypically, viral resistance to ACV is related to one of four mechanisms: complete deficiency in viral TK activity (TK deficient), decreased activity or production of low amounts of viral TK (TK low producer), viral TK protein with altered substrate specificity (TK altered), and viral DNA polymerase with altered substrate specificity (DNA pol altered) (Gaudreau *et al.,* 1998).

In this chapter single ACV resistant isolates from three HIV- positive patients and multiple sequential ACV resistant isolates from a BMT recipient were examined for phenotypic changes in the thymidine kinase and DNA polymerase enzymes which are known to be important in the mechanism of antiviral action of ACV. In addition, genotypic analysis of the TK enzyme was performed on all the viruses found to be resistant to the drug in vitro.

HIV positive patients at risk of severe disease with recurrent HSV infections and those being treated for active infection are normally treated with ACV often for prolonged periods. Swabs from herpetic lesions from these patients were routinely screened for the development of antiviral resistance as part of an earlier study. From this work, three isolates from separate patients were found to be drug resistant and were therefore subjected to further characterisation.

Assays for TK activity showed all three viruses to be TK negative, i.e. using the assays available at that time, no thymidine kinase activity was detected. However, genotyping suggests that a shortened protein sequence was only found in one of the three isolates. It is likely that this mutation resulted in a total absence of functional enzyme. Mutations such as the leucine to serine at amino acid 28 and the histidine to tyrosine change at amino acid 57 may also have given rise to a non-functional enzyme. Gaudreau *et al.,* (1998) have also reported single point mutations at codon 57, which is predicted to be within the ATP binding site of the enzyme. The authors also reported a number of base substitutions which gave rise to premature stop codons further downstream, with the result that shortened TK proteins were expressed. Although these authors did not test the proteins produced by the resistant viruses by Western blotting, other researchers have confirmed the production of shortened

proteins by this type of analysis (Sasadeusz *et al.,* 1997). In retrospect, this type of analysis for the resistant isolates reported in this study should also have been performed.

Detection of TK activity at low levels by existing techniques may lead to difficulty in discrimination between TK-deficient (TK^D) and TK-negative (TK-) phenotypes (Sasadeusz and Sacks, 1996), and it is possible that the assays used in this study were not sufficiently sensitive to correctly identify the low TK producers.

Resistant viruses were also recovered from six samples of oral lesions from a single BMT recipient, taken over a two week period while the patient was receiving ACV for prophylaxis against HSV recurrences. The first two apparently resistant samples from this patient had IC_{50} values of 4.8 and 4.0 μM ACV (sensitive strains normally have IC50 values between 0.1 - 1.5μM). These viruses were found to be positive for TK function, but borderline resistant when assayed for DNA polymerase activity. Although it is easy to see how the deletion of bases and subsequent frameshifts which result in shorter proteins may account for the lack of TK activity, the influence of single point mutations is harder to determine. Software packages are now available which allow the insertion of such changes into three-dimensional models of the enzyme, to predict the conformational alterations that might arise due to the properties of the different amino acids. This facility was not available to me at the time when these experiments were performed.

The isolate from sample 4f (lip lesion, see Table 6.3) had a lower IC50 value (16.12μM) than the previous three isolates from the same patient, but showed an altered TK activity and normal DNA polymerase activity. It may be that prolonged selective pressure <u>in vivo</u> facilitated the breakthrough of the more than one resistant virus in this patient. Alternatively, it is possible that the sampling and virus isolation methods used selected a virus with a TK altered phenotype because this virus grew better in the culture conditions than TK negative viruses also present within the lesion. Plaque purification of all resistant viruses would have provided a useful insight into the relative preponderance of multiple virus phenotypes within a single sample. Longitudinal studies in other immunocompromised patients may, however, reveal a

constantly fluctuating population of viruses, some of which exhibit resistant phenotypes and others which remain sensitive to drug.

The resistant isolates from Patients 1 and 2 were serotyped as HSV 2 when assayed using the monoclonal antibody typing kit, but sequence analysis of both the cloned DNA and direct sequenced PCR amplicons revealed an HSV 1 sequence. It is possible that these patients carried and reactivated more than one HSV infection and the isolates that grew through the ACV and were amplified by PCR were the type 1 viruses. Again, in patients where the possibility of isolating more than one virus strains is high, it would have been useful to have plaque purified viruses before subjecting them to PCR amplification and sequence analysis.

There were also a number of commonly seen mutations such as the amino acid change from leucine to proline at amino acid 42. Leucine and proline are both aliphatic amino acids, but proline differs from other amino acids in that its side chain is bonded to both nitrogen and α-carbon atoms, thus influencing the protein architecture. Additionally, unlike leucine, which has a typical hydrophobic aliphatic side-chain, proline is not averse to exposure to water. It is possible that the substitution of a proline in place of a leucine might alter the character of the TK protein sufficiently to abrogate enzyme activity. Computer aided analysis of these single amino acid changes in a three dimensional model can help to shed light on how the protein is altered.

Western blot analysis of the TK proteins would help to confirm the presence of truncated polypeptides caused by the deletion mutations, although plaque purification would be necessary to screen pure populations of mutant viruses.

The results from this chapter suggest that the presence of ACV resistant Herpes simplex virus may have resulted in the recurrence of herpetic lesions in some the immunocompromised patients described here. It is possible that increasing the dose of ACV and its for limited periods may prevent the emergence of resistant viruses, but this is not always possible in patients in prolonged states of immunosuppression. Other drugs are available for the treatment of ACV resistant virus strains. Mendel *et*

al., (1995), have observed that HSV TK deficient or TK altered mutants are more susceptible to Cidofovir (CDV) than to ACV in vitro. In some cases, it may be necessary to switch treatment to a pyrophosphate analogue such as foscarnet (Phosphonoformate, PFA), with does not require phosphorylation for activation.

In conclusion, an awareness of the problems associated with the breakthrough of ACV resistant viruses, such as increased transcription of HIV, may enable a more rapid alteration of therapy to CDV or PFA. In most cases, it is likely that re-population with ACV sensitive variants would occur rapidly in the absence of ACV.

Chapter 7

General Discussion

Immunocompromised patients are frequently at risk of severe, often life-threatening disease caused by CMV and HSV infections. For this thesis the role of these two viruses was examined in two groups of patients – those with HIV infection and in patients undergoing bone marrow transplantation.

Valaciclovir is a valine ester of aciclovir which is rapidly and almost completely converted to aciclovir after oral administration. In this way it achieves three to four-fold higher total plasma levels of drug than those achieved with ACV. VACV 2 g four times a day (qds), results in a total exposure of ACV similar to that achieved by i.v. ACV dosing at 10mg/kg three times daily, which is sufficient to inhibit many clinical strains of CMV (Cole and Balfour, 1987). High dose ACV has been shown to be effective at reducing CMV disease in bone marrow, renal and liver transplant recipients (Meyers et al, 1988; Balfour et al, 1989; Mollison et al, 1993). Although placebo controlled studies of ACV had previously shown a survival benefit in patients with advanced HIV disease, high dose ACV had not been shown to reduce the incidence of CMV disease (Cooper et al, 1993, Youle et al, 1994, Ioannidis et al, 1998). It is likely that using conventional ACV therapy in the HIV infected patient the levels of drug were not sufficiently high to have a substantial anti-CMV effect. In these studies, virus detection was achieved by culture methods, rather than the more sensitive PCR approach used in this thesis.

In Chapter 2 of this thesis qualitative PCR was used to monitor the presence of CMV in the blood and urine of HIV patients recruited onto the ACTG 204 study evaluating the efficacy of Valaciclovir. This was performed on 310 patients enrolled on the European and Australian arms of a virology substudy to determine the role of CMV DNAemia and viruria as potential markers for disease progression.

Patients were recruited onto one of three arms of the study comparing the anti-CMV efficacy of VACV with either high dose (800mg qds) or standard dose (400mg bid) of ACV. Because earlier studies had shown that ACV has no effect on CMV disease in the HIV patient, the two ACV doses in our VACV efficacy study were combined for analytical purposes. Patients who were PCR positive in blood or urine at trial entry were both at increased risk of CMV disease compared with patients who were PCR

negative. The blood PCR-positive patients were also 2.57 times more likely to develop disease at any time compared to those who were blood-PCR negative at baseline, irrespective of treatment allocation. But it was the patients who were blood PCR positive at trial entry who experienced the greatest benefit with VACV. Patients who were PCR positive in urine at baseline did not show a similar significant benefit with VACV treatment.

These results suggest that VACV was exerting its effect by reducing the virus load in the blood, an effect which may have been followed by a later reduction in the amount of virus excreted in urine. Further work to quantitate the viral load in the same patients has shown that viral load in both blood and urine in follow-up samples was reduced with VACV therapy (Emery et al, in press), with VACV resulting in a 1.3 log decrease in blood CMV load at 3 months on treatment. Thus, in viraemic patients with advanced HIV disease VACV delayed the onset of CMV end-organ disease. There was no survival advantage noted in patients allocated to either the VACV or the combined ACV arms, but, as would be expected, significantly higher proportion of patients who were blood PCR negative at trial entry survived to the end of the study period compared to patients who were baseline blood PCR positive. This feature was not seen with baseline urine PCR results. However, subsequent quantitative PCR analysis has shown that elevated viral loads in both blood and urine at baseline and throughout the study period were significantly associated with a faster progression to CMV disease endpoint, and in the case of blood, survival. In the studies presented in this thesis we have shown that detection of CMV in the blood by PCR provides a proximal marker for disease in patients at risk for CMV retinitis. This is similar to data obtained in a smaller study by Bowen et al, (1996), into the natural history of CMV in AIDS patients at risk for retinitis.

Although VACV treatment did not show a significant benefit in patients who were baseline urine PCR positive, a significant number of patients who were baseline urine PCR-negative remained negative throughout the study period, suggesting that this agent may be of use for CMV prophylaxis in the same way as high dose ACV is in transplant patients. A comparison of the results obtained from the 1654 trial of GCV (Spector et al, 1996) and the ACTG 204 study suggest that it is the bioavailability of

the antiviral drug which is important in individuals with systemic CMV infection rather than the inherent potency of the drug.

Clinical strains of CMV have IC50 values for ACV of between 16μM and 146μM (Cole et al, 1987), so high plasma concentrations need to be achieved before any anti-CMV effect is likely to be observed. Work on measuring the viral load in our patient cohort has shown that VACV achieves the required plasma drug level and thereby results in a reduction in CMV replication (Emery et al, in press). But quantitative PCR is time-consuming and expensive, so is it really necessary to have quantitative data to monitor the efficacy of the antiviral therapy? The qualitative PCR results I have obtained show that patients who are PCR positive in blood at baseline can become PCR negative with VACV treatment, and that baseline blood PCR negative patients can be protected from becoming PCR positive. So although having the quantitative PCR data is useful for determining the way in which the drug might be exerting the antiviral effect, sufficient prognostic and efficacy information can be gained by simply using qualitative PCR analysis on samples taken at reasonably frequent intervals i.e. at least monthly. Quantitative PCR assays are becoming easier to use, (e.g. the Roche AmpliCore assay, and the TaqMan assays), so in the future it may be feasible to run all PCR samples on a single, quantitative assay. Currently there are a number of commercial restrictions on the development and use of these assays for diagnosis of infection.

Although HAART has reduced the incidence of CMV disease in HIV patients, it is still important to monitor patients for "breakthrough" infections caused by failure of the HAART. Pre-emptive treatment with VACV may avoid the use of more toxic treatment with GCV, PFA or cidofovir. Alternatively, treatment strategies with combinations of these drugs and regular monitoring for antiviral resistance may be employed with qPCR information.

Because some clinical samples for PCR assays occasionally yield non-specific results, a confirmatory assay using an oligonucleotide probe conjugated to alkaline phosphatase was developed and evaluated for routine use with clinical samples (Chapter 3). Using this assay up to 68% of the samples which had yielded non-specific bands in the routine PCR assay were confirmed as being positive for CMV

gB DNA. Since the development of this assay, improved Taq polymerase enzymes have become available which produce significantly fewer non-specific results. Nevertheless, the confirmatory assay continues to be of use for the occasional sample producing non-specific amplification and the basic hybridisation technique can be used for a variety of PCR based assays for other DNA sequences. The assay has the additional advantage of providing a rapid and sensitive confirmation of PCR amplification, without the use of toxic ethidium bromide and an UV illuminator. An attempt was made to quantitate the viral DNA in blood using an adapted version of this assay, but assay results were not very reproducible. Further work is needed to optimise the quantitative probe based assay.

Since 1992, samples of blood and urine from immunocompromised patients have been routinely tested at our institution for the presence of CMV DNA using a qualitative PCR assay. In chapter 4 of this thesis I used quantitative PCR for the same CMV gB sequence to monitor fluctuations in viral load in the blood of BMT recipients. Longitudinal profiles of viral load in individual patients showing periods of antiviral treatment and clinical symptoms were plotted and analysed suing statistical methods. In this thesis I have shown that it is the viral load in the blood of the BMT patient is the single most significant risk factor for CMV disease. Viral load was significantly elevated in symptomatic patients compared to asymptomatic patients ($p = 0.002$). I have confirmed that the risk factor of recipient CMV seropositivity, which was identified in previous studies (Enright et al, 1993), is also significantly associated with disease, with seropositive recipients of seronegative marrow having a 6.6 times greater risk of disease than recipients of seropostive marrow. However, when the risk factors of viral load and recipient seropositivity were analysed in a multivariate statistical model, only viral load remained significantly associated with CMV disease, i.e. R+D- patients suffered disease because of a high viral load.

Although I have shown that elevated viral load is associated with CMV disease in the BMT recipient, and is important in understanding CMV pathogenesis, it may not be necessary to use quantitative PCR to begin antiviral treatment and monitor antiviral efficacy. Einsele et al, (1995), have shown that the use of qualitative-PCR guided

pre-emptive therapy significantly reduced the duration of antiviral intervention required while still very effectively reducing the incidence of CMV disease in patients treated with GCV. Therefore, by using PCR positivity to pre-emptively treat patients most at risk of disease, the duration of GCV treatment, which can be highly myelosuppressive, can be kept to a minimum while still maintaining effective antiviral therapy. Although the measurement of viral load provides a useful additional marker for monitoring the effficacy of treatment regimes, sufficient information may be obtained by using the qualitative PCR assay to treat most patients, providing that the PCR assay is used as a surveillance tool, and viraemia is detected early enough to reduce viral load before damage to target organs occurs. At the Royal Free Hospital, transplant patients are monitored at least once weekly for CMV viraemia by qualitative PCR and GCV therapy is now initiated on the basis of two consecutive positive results. In some instances, where clinical symptoms are indicative of an active CMV infection, GCV may be initiated following a single positive PCR result.

Immune reconstitution of CMV-specific CD8 CTLs and CD4 T helper cells has been shown to be important for the control of early (<100 days post-transplant) infection in the bone marrow recipient (Riddell, et al, 1992; Riddell et al, 1994). Patients receiving GCV therapy for early viraemia have a decreased proliferative response to CMV because of the myeloid toxicity associated with the drug (Riddell et al, 1994). Thus, PCR guided antiviral therapy, i.e., pre-emptive rather than prophylactic treatment, would allow a more rapid reconstitution of CMV specific T-cell responses and immune-mediated virus clearance.

There are a number of other complications of BMT that may predispose the patient to CMV disease. I examined the relative contribution to CMV infection, development of disease and overall survival by other potential risk factors, namely, the occurrence of Acute-GvHD; T-cell depletion; CMV strain variation; the recipients' HLA type; and the rates at which virus levels in the blood increased or decreased. These results are presented in Chapter 5 of this thesis. Studies of viral dynamics in the blood of the BMT patients showed no significant difference in the rate of clearance of virus in patients treated with GCV compared with patients who remained untreated. Indeed,

GCV treated patients experienced a slightly slower virus clearance rate compared to that of untreated patients, although this effect was not statistically significant. However, it should also be noted from the results presented in Chapter 4 that patients who were given GCV had higher peak levels of virus, and the rate of clearance may depend on the actual viral burden. It may also be the case that the myleosuppresion caused by the GCV may have reduced the natural, immune mediated clearance processes.

What is important is that viral dynamics data should be interpreted with caution, taking into account the probability that virus growth and decline may be a two component decrease, which may be influenced by an altered immune function and availability of target cells for the virus. Considerably more work, with more frequent sampling is required to allow a more accurate estimate of the virus dynamics in the blood of BMT and other organ transplant patients.

The association between CMV strain variation and disease has been studied in various immunocompetent and immunosuppressed patients (Fries et al, 1994; Shepp et al, 1996; Rasmussen et al, 1997). Since the gB protein is involved in the attachment of the virion to the cell membrane, and because the neutralising antibody response to CMV is primarily targeted at glycoprotein B, it is possible that variations in this region of the CMV genome may confer variability in the virulence and clearance of virus. In this Thesis, the blood samples from the BMT patients studied for longitudinal fluctuations in viral load were used to amplify a longer sequence of the gB gene for restriction enzyme based strain typing. Fifteen of the 49 patients who experienced CMV viraemia at any time during surveillance (see Chapter 4) were further characterised for gB strain variation. Of these, the largest proportion had type 2 infection (53%) followed by those with type 1 infection (33%). This finding was consistent with that obtained by other groups (Rasmussen et al, 1997). Because of the difficulty with amplification of the longer gB sequence, the number of typed strains was too small for adequate statistical analysis to correlate strain variation with clinical outcome. Optimisation of the PCR for this sequence proved to be very difficult, not least because the samples of extracted DNA had been frozen and thawed a number of times, and had been stored for up to five years, so a great deal of deterioration of the DNA might be expected. The previously described PCR assays for this sequence

were all optimised for Hirt extracted DNA from virus stocks grown to high titres in cell culture. Cell culture derived isolates were not available for this patient group because our laboratory has routinely used PCR assays for the diagnosis of CMV infection instead of cell culture for some years. Further studies in BMT and other patient cohorts are recommended, but not necessarily with cell culture amplified virus. If the gB sequence amplification can be optimised for virus that has not been passaged in cells, a more accurate picture of the viral populations might be obtained. There is a risk of detecting only cell-culture adapted strains if the virus is passaged before PCR. Restriction endonuclease analysis of a number of clones may also reveal the presence of more than one strain in any single compartment.

It is important to stress that isolates with the same gB genotype are not necessarily identical because genetic variations may occur elsewhere in the viral genome which could also affect tissue tropism and pathology. Efforts to define the genetic regions of CMV strains that are associated with virus pathology may provide important information that will help to explain the wide range of consequences of CMV infection in the immunocompromised host. To date, similar but limited studies on the membrane glycoprotein, gH, have shown no correlation between strain differences and disease (Fries et al, 1994).

Another major risk factor for CMV pneumonia after allogeneic BMT is the occurrence of acute GvHD. In order to reduce the incidence of A-GvHD monoclonal antibodies are used to deplete T lymphocytes from allogeneic marrow in vitro prior to transplant. My studies showed that significantly more individuals who received marrow with a lower level of T-cell depletion experienced A-GvHD ($p = 0.04$), and patients with A-GvHD also had a higher incidence of CMV disease. This increased risk was not, however, statistically significant ($p = 0.08$). In this group of patients there was no difference in the median peak virus loads between patients with or without A-GvHD. Purging the donor marrow of T-cells is a very effective means of reducing the risk of A-GvHD, but the risks of viral infections, tumour relapse and graft rejection are subsequently increased. One solution to this problem is to provide BMT patients with T-cells after transplant which are responsive to foreign antigens, but unresponsive to host HLA antigens. A method described by Rencher et al, (1996), involves the culture of donor T-cells with host derived B lymphoblastoid

cells, which serve to activate the T-cells which respond to the host HLA antigens. These activated cells are then removed using fluorescence activated cell sorting (FACS), leaving T-cell populations with helper and cytotoxic T cell responses against foreign antigens, but which are specifically unresponsive to host antigens. Thus patients benefit from T cell immunity and avoid the consequences of A-GvHD.
Studies using in vitro assays to determine the relationship between CMV specific T cell responses and CMV disease have shown that patients who recovered CMV specific CD8+ and CD4+ T cells were less likely to develop CMV disease than those who were deficient in these populations (Reusser et al, 1991; Li et al, 1994). Thus, adoptive transfer of T cells may be used to protect against CMV disease.

Early reactivation of CMV was found to increase the risk of interstitial pneumonia in patients given T-cell depleted marrow (Couriel et al, 1996). The authors recommend that patients receiving T-cell depleted marrow should be protected from CMV disease with prophylactic rather than pre-emptive treatments. However, prophylactic use of GCV is myelotoxic and results in delayed marrow engraftment, and severe disease after 100 days post-transplant is a significant problem. In our study all patients given allografts were given prophyaxis with ACV, with the option to increase ACV dosage or to introduce GCV on a pre-emptive basis.

Very little data is available for any association between CMV infection or disease and the HLA type of the BMT recipient. My analysis found no significant correlation between CMV infection or disease and any major HLA haplotype examined. Because of the small numbers, the analysis was performed on a limited number of Class I and II antigens only. Mismatches in the DR, DQ and minor histocompatibility loci may contribute to clinical outcome, but this analysis was not possible with this cohort of patients. Recent improvements in tissue matching of allograft recipients to their donors, together with improved antiviral therapy, and GvHD prophylaxis have greatly reduced the risk of mortality and morbidity due to graft mismatching.
However, I did find a significant correlation between HLA type B18 and an increased risk of early death post-transplant, whereas possession of haplotype B7 was associated with a reduced risk of early death. These patients did not experience any more CMV infection or disease than any other groups, and it is probably more likely that

possession of this haplotype is associated with more aggressive malignancies with poor prognosis even after BMT. Larger scale studies are necessary to elucidate the role of these haplotypes in CMV disease and survival in BMT patients.

HSV in the immunocompromised individual has the potential to develop into a disseminated infection which may result in morbidity and death if untreated. Prophylaxis against reactivation of the virus with ACV is routinely used for HIV infected and transplant recipients. Many patients are also treated with ACV for recurrences of oral and genital HSV infection. These patients often experience extended periods of exposure to the drug and are at increased risk for problems associated with drug resistant viruses. In this thesis, HSV isolates from immunocompromised patients treated with ACV were tested in vitro for drug sensitivity. Isolates which were found to be resistant in vitro were further characterised using phenotypic and genotypic analysis. Single samples from three HIV positive patients, and six sequential samples from a bone marrow transplant patient were found to have increased resistance to ACV in vitro. All the ACV resistant isolates derived from the HIV patients were negative for thymidine kinase activity and showed no loss of sensitivity in assays for DNA polymerase inhibition. Two of the early resistant isolates from the BMT patient showed some TK activity, but a low level of resistance at the DNA polymerase locus was also noted. A later isolate from this patient had a lower level of resistance to ACV in the in vitro assay and functional analysis of the enzyme revealed an altered TK activity. Sequence analysis of these isolates revealed a number of single base substitutions leading to coding changes which may have resulted in conformational changes to the enzyme structure, and therefore, altered binding affinities for the substrate. Single base deletions leading to predicted frame shifts and subsequent premature stop signals were also found in two of the isolates. These results are shown in Chapter 6 of this thesis. Hotspots for deletions and insertions of single bases have been reported recently (Sasadeusz et al, 1997), but in our samples these particular mutations in the TK gene were not observed. More accurate sequence information might have been obtained by using a number of additional approaches, such as the use of several internal sequencing primers reading both strands of the DNA, plaque purification of isolates to determine the presence of mixed virus populations, and Western blot

analysis to the expressed proteins to determine the presence of truncated polypeptides in mutants where this was predicted.

Although work on determining the mechanisms of ACV resistance has been going on for several years, regular monitoring of the incidence of resistance in the immunocompromised and the immunocompetent population is important if transmission of resistant virus is to be prevented.

In conclusion, this thesis has used PCR and other molecular tools to shed light on some of the mechanisms by which CMV causes disease in the bone marrow transplant recipient and in patients with advanced HIV infection. PCR analysis was used to gain insight into the efficacy of antiviral agents in patients enrolled on a clinical trial of VACV, and in bone marrow transplant patients treated with GCV on the basis of PCR results and/or clinical judgement. Further work to define other factors and immune mechanisms involved in the pathology of CMV infection is ongoing.

The genotypic and phenotypic changes in HSV resulting from prolonged ACV treatment were also determined, this data may add to the data recently published on the occurrence of resistance in both immuno-competant and –compromised patients. These data may allow an awareness of the potential problems of drug resistance in the immunocompromised host and facilitate the development of combination antiviral treatment to overcome or limit the problem of resistance.

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