Molecular and Serological Analysis of Herpesvirus Infections in the Immunocompromised Host

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Abstract

There are eight described human herpesviruses. All share the ability to achieve latency in their host following primary infection and may subsequently reactivate later in life. In immunocompetent individuals herpesvirus infections, especially those caused by reactivating virus, are usually mild however in patients with impaired cellular immune function they may cause significant morbidity and mortality.

In this thesis molecular and serological techniques were used to define the prevalence of the human herpesviruses in a variety of immunocompromised groups as these individuals are at greatest risk of severe herpesviral disease.

Human herpesvirus 8 (HHV8) is the most recently discovered of the human herpesviruses. A nested polymerase chain reaction (PCR) for detecting HHV8 genome was designed and used to examine the association between infection with HHV8 and HIV-associated Kaposi's sarcoma (KS). The prevalence of HHV8 genome carriage in the general UK population was defined, as were potential routes of HHV8 transmission. Novel treatments for HIV-KS were investigated.

The value of herpesviral genome detection in predicting associated disease was evaluated, together with the genome load response of cytomegalovirus (CMV), a significant herpesviral pathogen, to a novel chemotherapeutic protocol for immunosuppressed patients receiving allogenic bone marrow transplantation.

The prevalence and epidemiology of HHV8 were found to be compatible with that predicted for a causal agent of KS, but not the haematological malignancy multiple myeloma. Apart from HHV8, Epstein-Barr virus and CMV were found to be the most significant herpesvirus pathogens in the immunocompromised host.. The use of molecular detection techniques, such as PCR, were shown to be of great value in the diagnostic and epidemiological determination of herpesviral infection in immunocompromised patients.

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Abbreviations used in this thesis

A	= adenine
ACTG	= AIDS clinical trials group
ACV	= acyclovir
AIDS	= acquired immune deficiency syndrome
AZT	= azidothymidine (zidovudine)
BAL	= broncho-alveolar lavage
BCBL	= body-cavity-based lymphoma
BL	= Burkitt's lymphoma
BMT	= bone marrow transplant
BSA	= bovine serum albumin
bp	= base pairs
C	= cytosine
CD	= cluster differentiation
CDC	= Centre for Disease Control
cdks	= cyclin-dependant kinases
CMV	= human cytomegalovirus
CNS	= central nervous system
CPE	= cytopathic effect
CSF	= cerebrospinal fluid
CTL	= cytotoxic T-lymphocyte
DEAFF	= detection of early antigen fluorescent focii
ds	= double stranded
DNA	= deoxyribonucleic acid
EB	= extraction buffer
EBV	= Epstein-Barr virus
EBNA	= Epstein Barr nuclear antigen
ELISA	= enzyme-linked immunosorbent assay

ELONA = enzyme-linked oligonucleotide assay

EM = electron microscopy

FBS = foetal bovine serum

G = guanine

G+C = % molar content of guanine and cytosine

G-CSF = granulocyte colony stimulating factor

GCV = ganciclovir

GVHD = graft versus host disease

gp = glycoprotein

Hae III = *haemophilus aegyptii* isoenzyme III

HEL = human embryonic lung

HHV = human herpesvirus

HIV = human immunodeficiency virus

HLA = human leukocyte antigen

HRP = horse-radish peroxidase

HSV = herpes simplex virus

HVS = herpesvirus simairi

IE = immediate early

IF = immunofluorescence

IFN = interferon

IIF = indirect immunofluorescence

Ig = immunoglobulin

IL = interleukin

IM = infectious mononucleosis

IFN = interferon

i.v. = intravenous

IVDU = intravenous drug user

Kb = kilobase

kd = kilodalton

KS = Kaposi's sarcoma

KSHV = Kaposi'sarcoma-associated herpesvirus

LAT = latency-associated transcript

LB = lysis buffer

LMP = latent membrane protein

M = molar

Mab = monoclonal antibody

MCD = Multicentric Castleman's disease

mCP = minor capsid protein

MCP = major capsid protein

Mg^{2+} = magnesium ions

MGUS = monoclonal gammopathy of undetermined significance

MM = multiple myeloma

mPCR = multiplex polymerase chain reaction

NC = no clinical change

ND = not done

NHL = non-Hodgin's lymphoma

nm = nanometer

NO. = number

NPC = nasopharyngeal carcinoma

NS = no sample

OD = optical density

ORF = open reading frame

P = probability of null hypothesis (statistical anlysis)

PBMC = peripheral blood mononuclear cell(s)

PBS = phosphate buffered saline

PCP = *Pneumocystis carnii* pneumonia

PD = progressive disease

PCR = polymerase chain reaction

PD = probe diluent

PFA = phosponoformic acid (foscarnet)

PR = partial response

Pvu II = *Proteus vulgaris* isoenzyme II

RDA = repesentational difference analysis

RNA = ribonucleic acid

RPMI = Roswell Park Memorial Institute

sd = standard deviation

SD = sample diluent

SDS = sodium dodecyl sulphate

ss = single stranded

T = thymine

Taq = *Thermus thermophilus*

TIF = trans-induction factor

Tm = melt temperature

TK = thymidine kinase

TMB = 3,3',5,5'-tetramethylbenzidine

TNF = tumour necrosis factor

Tris = Tris (hydroxymethyl) methylamine

UCH = University College London Hospitals

UCLMS = University College London Medical School

UV = ultraviolet

VZV = Varicella zoster virus

v = viral

ZEBRA = Z-encoded Epstein-Barr replication activator

Section 1: Introduction

During the last two decades there has been a significant increase in the number of individuals receiving medical care who are defined as immunocompromised. The two most significant factors in this increase have been the use of immunosuppressive therapies in patients undergoing solid and bone marrow transplantation and the advent of the AIDS pandemic. Although fundamentally different in nature these two factors have resulted in a large number of individuals in whom the ability to combat effectively certain infectious agents has been reduced. Of particular concern in these patients are organisms which are commonly found in the majority of the general population including the commensal micro-flora and a number of viral types. These organisms are normally prevented from causing symptomatic disease by the actions of the host immune system, but in individuals lacking such immune control they can result in significant morbidity and mortality. The *herpesviridae* in particular have been associated with severe disease in the immunocompromised host. Usually causing mild, self-limiting disease in the human host they are associated with a number of severe and often life-threatening conditions in individuals with reduced immunological response.

This thesis is an investigation of the 8 human herpesviruses (table 1.1), in terms of their detection in the clinical setting and association with disease. The following sections describe the life cycle of these viruses and diseases with which each virus has been associated, especially in the immunocompromised patient. Where appropriate particular topics are highlighted as an introduction to work presented later in the text.

Table 1. 1. The Human Herpesviruses

Virus	sub-familly	trivial name	site of latency	genome size (Kb pairs)	Disease associations: primary infection	Disease associations: reactivation in immunocompromised
HHV-1	α	herpes simplex virus type 1	nerve ganglia	152	oral / genital lesions	severe form of primary lesions encephalitis
HHV-2	α	herpes simplex virus type 2	nerve ganglia	152	oral / genital lesions	severe form of primary lesions encephailitis
HHV-3	α	varicella zoster virus	nerve ganglia	125	chicken pox	shingles (in all individuals) retinal necrosis / encephalitis
HHV-4	γ	Epstein Barr virus	B lymphocytes	172	infectious mononucleosis	lymphoma
HHV-5	β	cytomegalovirus	leucocytes epithelial cells	229	cytomegalic inclusion disease	encephalitis / retinitis colitis / adrenalitis
HHV-6	β	human herpesvirus 6	T lymphocytes	162	roseola infantum	? encephalitis
HHV-7	β	human herpesvirus 7	T lymphocytes	145	unknown	unknown
HHV-8	γ	Kaposi's sarcoma-associated herpesvirus	B lymphocyte	185	unknown	Kaposi's sarcoma boby-cavity-lymphoma Castleman's disease

HHV - human herpesvirus

The herpesviruses are classified into three subfamilies; α, β and γ according to the virus host range and other biological properties (Roizman, 1990). α-herpesviruses grow rapidly in a range of tissues and usually destroy their host cells, β-herpesviruses grow slowly and only in a limited number of cells, while members of the γ-herpesvirus subfamily grow slowly in or immortalise the lymphoid cells of their natural host.

1. a. The *herpesviridae*

1. a. 1. Herpesviral genome structure and life cycle

Viruses of the herpesvirus family share a number of common features in both their physical characteristics and biological properties. All have genomes of double-stranded deoxyribose nucleic acid (DNA) (Roizman, 1993). These genomes are linear while contained within the virus particle and circularise to form episomes after infection of a susceptible cell and entry into the nucleus. Herpesvirus genomes, at between 130 and 230 Kb in size, are large in comparison with other DNA viruses (Roizman, 1993). The genomes have a conserved arrangement with sections of unique nucleotide sequence separated by multiply repeated shorter sequences. This type of genome arrangement is complex with up to six forms of gene organisation currently described within the virus family (Roizman, 1993). The presence and number of multiply-repeated genome sequences may vary and in consequence the size of individual genomes may alter by as much as 10 kilobases (Kb) in any one herpesvirus species (Roizman, 1993).

The mature virus particle is also complex in form; the genome is supported and attached to a protein core called the torus (Nazerian *et al.*, 1974). This torus-DNA complex is held within an icosahedral capsid consisting of 162 individual capsomer polypeptides, which is approximately 100nm in diameter (Wildy *et al.*, 1962). Surrounding the highly ordered capsid is an amorphous tegument region which is bounded by the outer layer of the particle; the envelope (Roizman *et al.*, 1974 and Epstein *et al.*, 1962). The envelope is a double membrane structure sequestered from the host plasma membrane during exit of the virion from the cell (Morgan *et al.*, 1959). Projecting through the viral membrane are a number of virus-encoded glycoproteins. It is these glycoproteins which facilitate the attachment of the virus to the cell and are major targets for host immunological response (Nemerow *et al.*, 1989 and

Marshall *et al.*, 1992). The overall size of the herpesvirus particle may vary from between 120 - 200 nm (figure 1a).

Cellular infection begins with attachment of the virion to the cell surface. A number of cell-surface proteins have been described as potential sites of virus interaction, including heparin and the fibroblast growth factor receptor for herpes simplexvirus 1 (HSV-1), and the CD 21 molecule of B-lymphocytes for Epstein Barr virus (EBV) (Shieh *et al.*, 1992 and Li *et al.*, 1992). Pre-incubation of cells with monoclonal antibodies against these proteins has been shown to inhibit viral infection (Fingeroth *et al.*, 1984). One, or more, of a range of herpesvirus glycoproteins may be involved in cell-surface / virus attachment, including, for HSV-1 gD, gH and gL, and for EBV glycoprotein (gp) 350 / 220. Following attachment of the virus to the cell surface an extremely rapid fusion process occurs between the viral envelope and the cell plasma membrane. Phagocytosis seems to be of little importance in herpesviral cell entry (Huang *et al.*, 1964). Naked virion capsids are then transported to the cell nuclear pores, via the cellular cytoskeleton, where the viral DNA is released into the nucleus (Dales *et al.*, 1973).

After shutting off host macromolecular synthesis herpesviral transcription occurs in the host cell nucleus, with the subsequent translation by cellular proteins taking place in the cytoplasm. Herpesviral protein synthesis occurs via a controlled cascade system with each temporal set of proteins synthesised regulating the production of subsequent viral gene products (Stamminger *et al.*, 1990). The immediate-early genes (α-genes) are the first to be expressed. Most α-gene-products have been found to have regulatory functions and begin to be synthesised approximately 2 - 4 hours post-infection. A failure in production of these proteins results in failure to produce the early gene (β-gene) products responsible for the organisation of viral DNA replication. Viral DNA replication, by the "rolling-circle" method, is mediated by a group of 7 virus-encoded polypeptides including a DNA polymerase, a single-strand DNA binding protein and a protein complex which has primase and helicase

Figure 1. a. Schematic representation of a mature herpesvirus virion

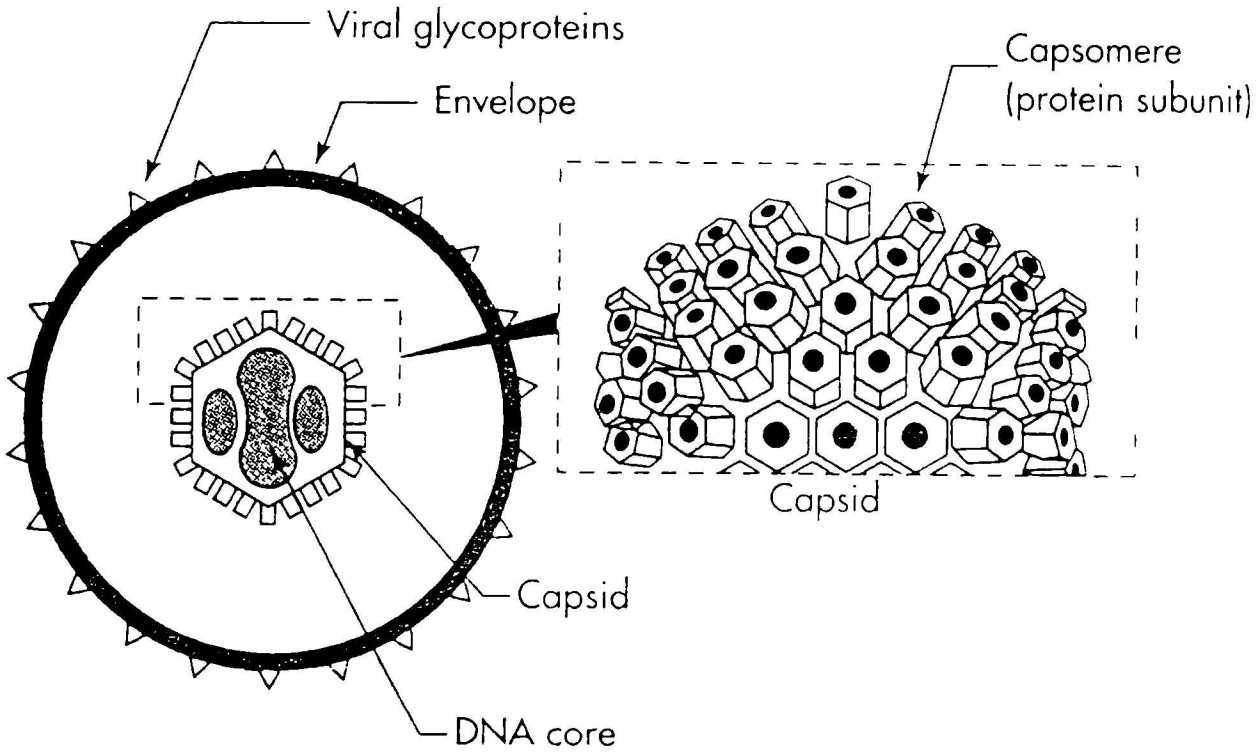
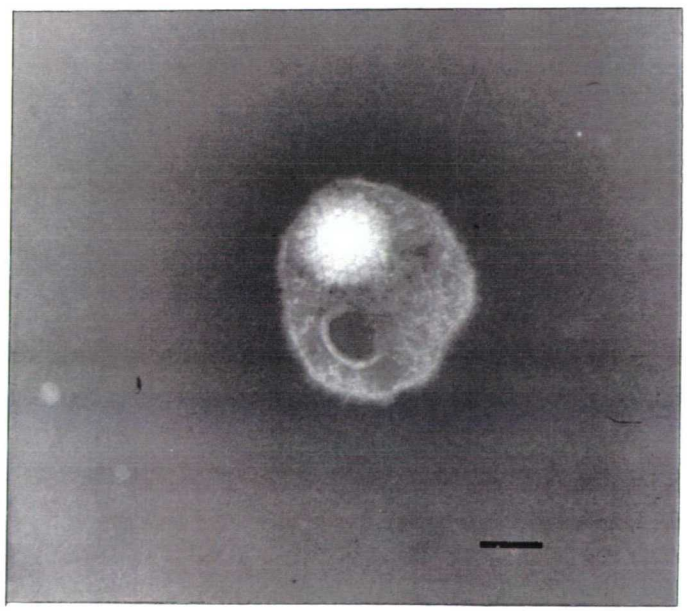


Figure 1. b. Negatively-stained herpesvirus particle (varicella-zoster virus) examined by electron-microscopy. Photograph supplied by Mr. Steven Rice, Department of Virology, UCLMS. Bar = 100nm.



functions (Challberg *et al.*, 1986 and Crute *et al.*, 1989). The appearance of the viral β-gene products signals the onset of viral genome replication. The final group of herpesvirus gene products are encoded by the late genes (γ genes), of which the most important and prevalent is the major capsid protein (ICP5 in HSV-1, p150 in EBV and UL86 in CMV all of which have significant intervirus homology: Dolyniuk *et al.*, 1976 and Chee *et al.*, 1989). It is believed that the capsid protein, although a γ-gene product, is produced throughout the cascade sequence but its concentration increases dramatically during and after DNA replication (Roizman, 1993). Following production in the cell cytoplasm capsid proteins are amalgamated into empty capsids and transported to the nucleus where the newly-synthesised, concantermeric, viral DNA is processed and inserted (Ladin *et al.*, 1982).

After construction of the viral capsid and introduction of the viral genome the progeny particles are transported to the cell surface where they are enveloped in the cell membrane. Prior to this envelopment the synthesised viral glycoproteins are processed cytoplasmicaly (Campadelli-Fiume *et al.*, 1985). After passage through the plasma membrane and acquisition of these membrane-based glycoproteins the progeny virions are released into the extracellular environment by reverse endocytosis displaying a typical morphology common to all members of the herpesvirus family (figure 1b).

As well as having a regular physical structure herpesviruses share two properties which may be considered characteristics of members of the family. Firstly they are widespread in the animal kingdom, with over 100 animal species having at least one associated herpesvirus (Roizman, 1993). Amongst humans eight distinct species of herpesvirus have already been discovered most of which are present in much of the world's populations, with little regard of race, geographical origin or socioeconomic status. Secondly, all members of the family are able to establish latency in infected cells. This is the ability of these viruses, following infection of a cell, to not only subvert the host cellular machinery and produce new virions; the lytic cycle of virus

replication, but also to persist indefinitely in the infected cell (see section 1. a. 2.) (Baringer *et al.*, 1976 and Henderson *et al.*, 1977). The majority of individuals who have been infected by a herpesvirus will continue to carry the virus in its latent state for life, occasionally experiencing brief periods of viral emergence or low level chronic replication. It is the high incidence of herpesviruses in the population together with their ability to reactivate from latency, replicate to high titre and cause disease in the absence of effective immunological control which makes this group of viruses of such clinical significance in the immunocompromised host.

1. a. 2. Herpesviral latency

Crucial to the understanding of the clinical consequences of herpesvirus infection in man is the process of virus latency. It would appear that all human herpesviruses can achieve latency in certain cell-types allowing evasion of host immune surveillance and subsequent reactivation and replication at a later time. In this section the current theories of viral latency as they apply to the neurotropic herpesviruses HSV-1 and HSV-2 as well as the lymphotrophic herpesviruses EBV and human herpesvirus 8 (HHV8) are examined.

Latency may be divided into a number of stages, each of which appear highly controlled but are not currently well understood. The first process in the establishment of latency is the primary infection of susceptible cells. It is important at this stage that the virus does not enter into its cascade of gene-product translation as this will result in cytocidal death of the cell (Birmanns *et al.*, 1993). For HSV-1 the site of latency is the body of sensory neuronal cells (Rock *et al.*, 1983). Once the virus has reached this cell site the α-gene trans-induction factor (α-TIF), a structural part of the virion, would usually interact with the de-encapsulated viral DNA to initiate the transcription cascade (Post *et al.*, 1981). However, by binding with the cellular transcription factor Oct-2 it is thought that such interaction is inhibited and the cascade

arrested before production of the ICP0 gene product. ICP0 is a nuclear phosphoprotein which acts as the initiator of the HSV lytic cycle protein cascade (Jordan and Schaffer, 1997). In this latent condition the viral DNA is maintained as a circular molecule in the nucleus in an episomal state. During latency none of the usual α, β or γ genes are translated, instead two types of latency-associated transcripts (LAT) are produced (Stevens *et al.*, 1987 and Rock *et al.*, 1987). The two most abundant forms of LAT are 1.5 and 2.0 Kb in size with the smaller transcript derived from the larger by post-transcriptional splicing. The functions of these transcripts are still to be fully determined but as they are sequence-complementary to the ICP0 gene, and overlap that site, they may act to maintain latency by anti-sense action preventing ICP0 transcription (Rock *et al.*, 1987). As no HSV viral proteins have yet been detected during the state of latency it would appear that by whatever action these LAT's exert their activity it is they alone which ensure the continuance of the viral latent state.

Mechanisms by which the latent HSV genome can reactivate are also currently poorly understood. It is known that a variety of stimuli are capable of initiating reactivation including; local tissue damage, unassociated host illness, a reduction in the host immune level and irradiation with ultra-violet light (Spruance *et al.*, 1985 and Muller *et al.*, 1972). The actual molecular mechanisms required for production of ICP0 and entry into the cascade of viral polypeptide production are unclear, although it has been suggested that the latent genome load in a cell nucleus may influence the ease by which the latent state is broken (Roizman, 1993).

The γ-herpesviruses EBV and HHV8 appear to exhibit a number of differences from HSV in the establishment and maintenance of latency and, rather than their site of latency being the bodies of sensory neuronal cells, both these viruses are able to become latent in peripheral blood B lymphocytes (Tosato *et al.*, 1985). The establishment of EBV latency results in the immortalisation of those latently infected cells (Sudgen *et al.*, 1977) and, unlike HSV, a larger

portfolio of viral transcripts are produced by EBV during latency. In common with HSV two small non-polyadenylated RNA molecules are transcribed and may be present at extremely high copy number, up to 10^7 copies per infected nucleus (Howe *et al.*, 1986). There have been two proposed functions of these transcripts, although *in vitro* data is far from conclusive. They may have effect by directly inhibiting the activation of a cellular interferon-induced protein kinase. This would cause a block in cellular translation by preventing phosphorylation of the protein synthesis factor eIF-2 alpha, thus stopping EBV protein production. Alternatively they may exert influence by acting as RNA-splicing catalysts for the post-transcriptional processing of the Epstein Barr viral nuclear antigen (EBNA) complex. The EBNA complex was first detected by immunofluorescence and has been found in the nucleus of EBV latently-infected cells (Klein *et al.*, 1974). The complex is now known to consist of at least six proteins but their individual functions are not all clearly understood. EBNA 1 appears to have a primary function of maintaining the episomal state of the EBV genome during latency (in contrast to the inert genome of HSV during latency) and exerts its activity by binding to the latent replication origin of the viral genome (Rawlins *et al.*, 1985). From studies of a variety of EBV-associated conditions EBNA 1 would appear to be the only viral protein which is uniformly expressed in all EBV-infected cells. EBNA 2 is a transcription factor but interacts with the cellular protein RBK-Jκ rather than directly with viral or cellular DNA. Its role, if any, in latency is undefined but it appears to be important in the up-regulation of certain cellular proteins, including the B-cell activation protein, CD23, which in soluble form has been found to act as a growth factor for B cells and to up-regulate the production of the EBV receptor protein CD21 (Wang *et al.*, 1987). It is the antigenicity of the EBNA 2 gene product which defines the two sub-types of EBV; EBV 1 which is more common in Western populations and EBV 2 which is prevalent in central Africa. The family of EBNA 3 genes are also type-specific and as well as causing up-regulation of CD21, the EBV receptor, modulate the transcription of the EBV latent membrane protein (LMP).

Also expressed during EBV latency are the EBNA leader protein and the two LMP's. Functions of the EBNA leader protein are unknown although co-localisation with the cellular retinoblastoma protein and p53 has been noted (Mannick *et al.,* 1995). LMP 1 is found in the cell membrane and has been shown to have a direct cellular transforming ability in epithelial cell lines as well as inducing cellular anti-apoptotic genes, including bcl-2, thereby supporting cell immortalisation (Nicholson *et al.,* 1997). LMP 1 effects on cellular expression are mediated through the cellular NFκB transcription factors and through direct interaction with the cellular tumour necrosis factor receptor-associated factors (TRAF) which are responsible for cell cycle and apoptosis regulation. Studies using EBV mutants which produce truncated LMP 1 gene products, unable to bind TRAF, have shown that this interaction is an absolute requirement for EBV-transformation of primary B-lymphocytes (Izumi *et al.,* 1997). The second membrane protein, LMP 2, co-localises with LMP 1 in patches on the cell membrane (Longnecker *et al.,* 1990) and is associated with the lyn-tyrosine kinase. The association would appear to block activation of infected B-cells by interfering with the signaling pathway from activated immunoglobulin receptors and is thought to be crucial to maintenance of EBV in its latent state (reviewed in Roizman, 1993).

As can be seen information on the molecular processes by which EBV maintains latency in immortalised B-lymphocytes remains fragmented. Even less is known about the entry of HHV8 into latency. Currently two RNA transcripts have been detected in HHV8 latently-infected B cells. One of these 0.7 Kb in size (T0.7) has been found difficult to express as a protein, the other 1.1 Kb (T1.1) in size has no perceived translational form (Staskus *et al.,* 1997). Neither of these transcripts have any known function but may both be commonly detected in latently infected cells, although T1.1 also appears to be an important factor in HHV8 lytic replication. Currently only one latency-associated nuclear protein has been described in HHV8-infected cells, the HHV8 latent nuclear protein (LNP) (Rainbow *et al.,* 1997). This protein, of 230 Kd, is encoded by a region of the HHV8 genome which shows poor

homology with other γ-herpesviruses and any functional similarities between LNP and the EBV EBNA proteins is undetermined at this time (Neipel *et al.*, 1997).

Transcriptional activation from the latent state by the γ-herpesviruses is also poorly described, however studies have highlighted the importance of the Z-encoded Epstein-Barr replication activator, or ZEBRA protein in EBV (Miller 1990). This is a viral switch protein encoded in the BZLF1 gene of EBV which acts as a transcriptional activator to allow production of the EBV α-gene products, including possibly ICP0* and the viral DNA polymerase (Miller 1990). Activation of these genes re-initiates the cascade system of lytic virus replication with the viral polymerase producing new linear genomes which may then be packaged into naked virion capsids. The detection of ZEBRA is a definitive marker of permissive, lytic infection in the cell. Unfortunately the HHV8 genome does not appear to have a genome sequence with homology to the BZLF1 gene, although it does possess a homologue to the EBV BRLF1 transactivator which has also been postulated to have role in viral reactivation (Russo et al.1996, Zalanai *et al.*, 1996).

* the analogue of HSV-1 ICP0

1. a. 3. Herpes simplex viruses

There are two closely related, but distinct, types of herpes simplex virus; type 1 (HSV-1) and type 2 (HSV-2). Both have been classified as α-herpesviruses, this designation relates to their variable host range, short replicative cycle both *in vivo* and *in vitro,* and capacity to establish latency in sensory ganglia (reviewed in Steiner, 1996). As well as these factors these two closely-related herpesviruses share many biological properties, including their preference for initial infection via mucosal epithelium, either orally (predominantly HSV-1) or ano-genitally (predominantly HSV-2) (Juretic *et al.,* 1966). The HSV-1 and HSV-2 encoded proteins have been extensively studied (Knipe, 1989 and Roizman and Sears, 1990) with two (gB and gH) having homologues in all other members of the human herpesvirus subfamily.

During primary HSV infection shallow fragile vesicles, which rapidly ulcerate, can be seen in the involved mucous membranes. These are associated with localised pain, itching, swelling and discharge. Systemic symptoms of fever and malaise are also common. After entry into the host and systemic spread the virions are transferred by retrograde axonal transport to the dorsal root ganglia where life-long latency is established (see section 1. a. 2.). From this site of latency there may be periodic bouts of reactivation which usually only cause the local symptoms of primary infection but do allow for horizontal virus transfer to susceptible contacts (Gesario *et al.,* 1969). Such reactivation is common following unrelated illness, fatigue and immunosuppression. For both the types transmission may be via infected saliva, , cervical secretions or infected genital epithelium. By the end of pubescence over 50% of most world populations have been infected with either or both types of HSV (Wentworth *et al.,* 1971).

HSV infections are usually minor clinical events requiring no specific treatment and resolving through the actions of the host immune system,

particularly the CD8+ve T cell-mediated immunity, within a few weeks (reviewed in Roizman, 1993). However in more susceptible individuals, such as the immunocompromised host, HSV infection, both primary, and as a result of viral reactivation, may result in widespread, systemic, disease. In neonates transmission from the mother may occur either *in utero* or more commonly at birth during passage through the HSV-infected vagina. Symptoms can range from localised eye and skin lesions to involvement of the brain in the form of an encephalopathy and a life-threatening systemic infection affecting multiple organ systems including the central nervous system (CNS), lungs, liver, adrenals, skin, eye and mouth. In the systemic form of neonatal HSV infection up to an 80% case-fatality rate has been recorded, with survivors frequently suffering from long-term neurological sequelae (Whitley *et al.*, 1985). Such a spectrum of disease is also seen in the adult immunocompromised host where, however, the source of virus is most likely to be from the reactivation of latent HSV following a previous infection, rather than from a primary infection. Such reactivation leads to viral transfer by axonal transport to the sites of primary infection where viral replication may cause extensive genital and oro-facial lesions which are both painful and disfiguring (Whitley *et al.*, 1984 and Mann *et al.*, 1984). A range of internal organ sites may also become affected by HSV infection, most severely the brain and CNS where infection, in these individuals, may lead to a number of central and peripheral neurological symptoms associated with damage to the temporal lobes and meninges. The incidence of HSV-related neurological disease in the immunocompromised host is not thought to be significantly higher than in the general population, however in patients with severe immunodeficiency its course may be protracted and the sequelae more pronounced (Schiff *et al.*, 1998).

Treatment of individuals with severe HSV infection is with the anti-herpesvirus chemotheraputic acyclovir (ACV), a nucleoside analogue inhibitor of HSV genome replication. To be active against HSV ACV first requires triphosphorylation. The conversion to the monophosphate form is mediated by the action of a viral thymidine kinase (TK) enzyme. Cellular kinases then

complete the phosphorylation producing the active ACV-triphosphate. The triphosphorylated form of acyclovir acts as a DNA chain terminator by incorporation into the newly-formed lengths of HSV genome synthesised by the HSV-encoded polymerase enzyme (Elion *et al.*, 1977). Its effectiveness is due to the 100-fold higher affinity of the drug for the HSV-encoded polymerase in comparison to the host cell polymerase, as well as an absolute requirement of the viraly-encoded thymidine kinase for drug monophosphorylation. Both these factors allow high plasma levels of ACV to be tolerated in the patient without the development of undue toxicity. Indeed in certain immunocompromised patients, such as those experiencing severe T-cell mediated immune dysfunction because of HIV-infection or due to radiological preparation for organ transplantation, acyclovir can be given for prolonged periods as a prophylactic therapy to prevent HSV and cytomegalovirus (see section 1. a. 6.) reactivation and disease (Wolf *et al.*, 1993). Although not usually necessary for the self-limiting disease seen in the immunocompetent host ACV has been a highly effective drug in the treatment of HSV infections in the immunocompromised. The use of conventional and multiplex PCR for the diagnosis of HSV infection in the CNS and ocular sites of HIV-infected patients is discussed in sections 3. e. and 3. f.. Such molecular detection methodologies have significantly decreased the time required to confirm a diagnosis of cutaneous and / or systemic HSV infection and initiate appropriate ACV therapy.

1. a. 4. Varicella-Zoster virus

In common with the two herpes simplex viruses varicella-zoster virus (VZV) is an α-herpesvirus which shows significant homology in gene layout with HSV-1 and HSV-2 (Davidson and Scott, 1986). VZV is globally widespread, with the majority of infection occurring before puberty (Preblud *et al.*, 1984). Two distinct patterns of disease are associated with VZV, depending on whether the infection is of a primary or reactivative nature. Primary infection most

often occurs by inhalation of expelled droplets containing virus particles and infection of the mucosa of the upper respiratory tract and oropharynx. From this site there is rapid dissemination of the virus through the body via the blood and lymphatic systems. When the virus reaches the epithelial cells of the skin focal lesions develop, containing high titres of virus (10^9 ml^{-1}), which form the characteristic "chicken-pox" rash. Usually this disease is mild and the host immune-system terminates the viremic phase in approximately 3 days, after which no new lesions are seen (Asano *et al.*, 1985). Disease associated with primary infection may be significantly more severe when acquired *in utero* during the first trimester of pregnancy and in adults past the age of pubescence (Feldman *et al.*, 1975 and Stagno and Whitley, 1985). Occasionally in these individuals lesions can be found in the mucosa of the respiratory, gastrointestinal and genitourinary tracts and the parenchymal membrane of almost all organs. In these patients varicella-associated pneumonia is the most serious consequence of infection. Infection and direct damage to the central nervous system (CNS) has been described and is responsible for about 20% of chicken pox-associated hospitalisations in children aged 5 - 14 (Fleisher *et al.*, 1981).

As is characteristic with all the herpesvirus infections VZV will enter a latent phase following primary infection. The sight of latency are the dorsal sensory ganglia where the virus remains quiescent. Various factors have been associated with viral reactivation. As for HSV most of these have the effect of temporarily lowering host immune function, particularly the cell-mediated immunity, and it is believed that it is this drop in immunological control which contributes to the re-commencement of VZV replication in the sensory ganglion. Virus particles then pass down the sensory nerves sometimes producing neuronal necrosis and inflammation until reaching the skin, where a number of clustered vesicles form in a dermatomal pattern closely associated with the underlying nerve cells down which the virus has traveled (Straus *et al.*, 1984). These vesicles are the visual manifestation of VZV reactivation; known as herpes zoster. Such zoster lesions will generally heal quickly in a

similar manner to those produced during chicken pox although there may be an associated post herpetic neuralgia especially in patients over the age of 60. This neuralgia may be prolonged even if ACV therapy has been initiated during the herpes zoster episode. A less common site of zoster is the eye and associated skin. Occurring in approximately 15% of patients this is the result of virus transfer down the ophthalmic division of the trigeminal nerve (Ragozzino *et al.*, 1982). Morbidity arising from this complication demands ACV therapy.

The severity of VZV infection may be significantly increased in the immunocompromised host, especially in those experiencing a primary infection, where persistent viremia may result in severe cutaneous zoster as well as involvement of the lungs, liver and CNS. These symptoms can be particularly serious and potentially fatal in paediatric patients with an underlying immunodeficiency who have lost immune protection from maternal antibodies. However, in general, VZV disease due to reactivation in adults with severe immunodeficiency can be effectively treated with high dose intravenous ACV and is not associated with significant levels of patient mortality (Gelb *et al.*, 1990). The multiplex PCR methodology described in section 3. f. includes reagents for the detection of VZV DNA. Commonly CNS and ocular fluids from immunocompromised patients would be analysed by this method to determine the presence of VZV genome allowing rapid intervention with ACV.

1. a. 5. Epstein-Barr Virus

Epstein-Barr virus (EBV), a γ-herpesvirus, has markedly different biological properties to those of the α-herpesviruses described above and is more closely related at the genomic level to a number of old world monkey herpesviruses such as herpesvirus saimiri (HVS) (Baer *et al.*, 1984). All of these γ-herpesviruses have a restricted host cell range and replicate predominantly in

immunoglobulin-producing B lymphocytes, the site where latency is also established. Following latency a group of viraly-encoded proteins; the EBV nuclear antigens (EBNA's) are produced both to maintain the state of latency and, in the case of EBNA-2 induce B-cell immortalisation, a well-described characteristic of EBV infection (see section 1. a. 2.). As well as B-cells EBV has also been found to infect some types of epithelioid and fibroblastic cells, where lytic infection occurs with the production of projeny virions and cell death (Sixbey *et al.*, 1984). Attachment of EBV to susceptible cells is mediated by the virus-encoded gp 350 / 220 complex with the C3d complement receptor, CR2, of cells (Fingeroth *et* al., 1984). It is the epithelial cells of the oropharynx which are commonly the site of primary infection and may support a continuing low level of persistent replication and pharyngeal shedding for extended periods of time allowing efficient transfer to other susceptible individuals (Gerber *et al.*, 1972 and Allday and Crawford, 1988). Most transmission of EBV is via infected saliva and usually occurs during social contact early in life, with little associated disease, resulting in a seroprevalence of 80-90% in most populations by early adulthood (Crawford, 1994). However, if a susceptible individual is infected with EBV late in childhood or during adolescence a range of clinical symptoms may then occur including sore throat, fever, headache and malaise and are given the general name infectious mononucleosis (IM), or glandular fever (Henle and Henle, 1986 and Neiderman *et al.*, 1970). Such symptoms may be due to the cytotoxic T-cell response the body mounts to EBV-infected B lymphocytes. Complications of IM such as jaundice, myalgia and neurological symptoms are rare and although there have been reports of fatal lymphoproliferative disease in pregnant women with IM there are few reports of foetal damage during maternal primary infection (Straus *et al.*, 1985 and Hotchin *et al.*, 1989). Most fatalities associated with IM have occurred in individuals with X-linked lymphoproliferative syndrome (Crawford, 1994) as such patients are unable to mount a cell-mediated immune response to EBV infection.

The range of EBV-associated disease in immunocompetent populations has been found to differ dramatically depending upon geographical location. In western Europe, Australasia and the America s IM is by far the most usual clinical consequence of EBV infection, however in equatorial Africa Burkitt's Lymphoma (BL) is a common EBV-associated childhood malignancy which accounts for nearly 80% of all childhood cancers in the area (Burkitt *et al.*, 1958). The general incidence of BL is 1 case per 3000 children during the first 15 years of life, with one prospective study in Uganda of 42,000 children showing that 12 developed BL (The *et al.*, 1978). Because of the restricted geographical nature of BL associated co-factors have been suggested including malnutrition and the high local incidence of malaria. It has been postulated that EBV-infection stimulates B-cell division and hence the number of infected B-cells, an undefined co-factor (probably co-incidental malaria) then impairs T-cell capacity to control proliferation of the immortalised B-cells (in an analogous manner to the X-linked lymphoproliferative syndrome) in which activation of the c-*myc* oncogene is common. This sequence of cell cycle control failure then leads to the establishment of lymphatomous tissue (reviewed in Roizman, 1993). BL was also one of the first tumors shown to have a characteristic chromosomal translocation involving chromosome 8 near the location of the *c-myc* oncogene and a site on either chromosome 14 or 22. This rearrangement alters the regulation of expression of *c*-myc by placing it under the control of a strong immunoglobulin gene promoter present in chromosome 22 (Lenoir *et al.*, 1982). All African BL tumours contain EBV DNA in each malignant cell and in every tumor the latent EBV episomal genome has been found to be clonal indicating tumour generation from a single EBV-infected cell (Nonoyama *et al.*, 1973). The latency-associated protein EBNA 1 has also been consistently detected in BL tissue (Rymo *et al.*, 1979).

In southern China nasopharyngeal carcinoma (NPC) is another EBV-associated tumor which is over 50 times more common in that population than in European and north American Caucasians (Ho *et al.*, 1978). In a similar

manner to African BL EBV genomes have been found in all NPC-associated cells and the tumours have been shown to be clonal in origin (Wolfe *et al.*, 1973). However in contrast to the lymphoid BL in NPC, an epithelial tumour, not only is EBNA 1 expressed sotoo are LMP 1 and 2 as well as the EBER RNA transcripts (Brooks *et al.*, 1992). No *c*-myc rearrangement has been noted in NPC. Other co-factors which are thought to be required for disease development include a genetic susceptibility, possibly HLA-driven, and environmental factors including local diet. Recently it has been shown that the EBV positive NPC-transformed cells appear able to avoid cytotoxic T-cell immunological responses (Tsukuda *et al.*, 1993). It is thought that an, as yet, unidentified viral protein inhibits viral antigen presentation and thereby circumvents immunological response allowing development of the tumour.

In the immunocompromised host, especially those who are HIV-infected, EBV has been found to be present in a much greater percentage of peripheral blood cells than in normal EBV-seropositive individuals (Birx *et al.*, 1986). In section 3. a. 4. detection of EBV DNA in the peripheral blood of HIV-infected individuals is analysed as a control in determining the proposed association of the other γ-human herpesvirus, HHV8, with Kaposi's sarcoma. The increased systemic titre of EBV DNA in immunocompromised patients is probably a reflection of the reduction of immune surveillance in these patients and consequent increase in EBV replication. The increase in EBV viral load is also paralleled by the number of EBV-associated diseases found in the immunocompromised host. These are generally known as progressive lymphoproliferative disorders. Such diseases have a wide spectrum of severity, from inconvenient to life-threatening. Oral hairy leukoplakia (OHL) was at first thought to be a direct result of HIV-infection as it was almost completely restricted to HIV-seropositive individuals. Subsequently OHL has been shown by *in situ* hybridisation to be EBV-related and manifests as a bilateral white lesion of the tongue of usually no more than cosmetic significance (Neidobitek *et al.*, 1992). Of far more clinical importance is the high incidence of non-Hodgkin lymphoma (NHL) in immunosupressed individuals (Levine *et al.*,

1990). AIDS-related NHL probably occurs after the loss of function of CD4+ve helper lymphocytes and is due to the immortalisation and uncontrolled replication of EBV-infected B-cells. It is described as an "end-stage" disease, i.e. occurring when immune status, as defined by CD4 T-lymphocyte count, has fallen from the norm of 1200-1500 to below 200. NHL is actually a spectrum of disease which ranges from polyclonal lymphoproliferation to monoclonal lymphoma, all of which may have systemic involvement, most AIDS-related NHL's are high-grade with approximately 60% being B-cell immunoblastic in type. Although EBV can be detected in the majority of NHL types, other factors such as re-arrangement of the *c-myc* gene have also been found to be associated, in a similar fashion to African BL (Bhiata *et al.*, 1994). As well as in systemic NHL EBV has been found in virtually all AIDS NHL presenting as primary central nervous system (CNS) lymphoma (MacMahon *et al.*, 1991). Almost 40% of AIDS-NHL have CNS involvement. Although response to therapy for CNS lymphoma, involving combination chemotherapy and radiotherapy is noted, in a large percentage of patients median survival is less than six months due to the frequent uncontrolled relapse of disease.

Patients experiencing acute immunosuppression following solid organ, or bone marrow, transplantation are prone to a spectrum of post-transplant lymphoproliferative diseases associated with EBV infection (especially primary infection), (Crawford *et al.*, 1980 and Langnas *et al.*, 1991). These are characterised by an unregulated expansion of EBV-immortalised B-cells and most have been shown to be clonal in origin (reviewed in Nalesnik, 1996). As with the HIV-infected individuals immunosuppressed transplant patients have a poor prognosis with such disease, and although initially responsive to chemotherapy commonly experience only a short duration of remission. In fact in these non-HIV-infected patients the most effective "treatment" appears to be a return of the patient to a normal immune status as rapidly as possible. This allows restored functioning of the individuals cytotoxic T-cell (CTL) response which, in contrast to NPC, is effective against the EBV-infected

lymphocytes due to their full expression of all EBV-latent proteins (Murry *et al.*, 1988). Immune function can be restored by the reduction of immunosuppressive therapy used in such patients although this may not always be possible as reduction often results in subsequent rejection of the transplanted organ. Alternatively studies have recently begun on the therapeutic effectiveness of EBV-specific CTL infusions in patients for whom a return to normal immunological status is impossible (O'Reilly *et al.*, 1997 andHeslopand Rooney, 1997).

1. a. 6. Cytomegalovirus

Human cytomegalovirus (CMV) is a β-herpesvirus with a highly complex genome showing a number of regions with extensive homology with human DNA sequences (Ruger *et al.*, 1984). The only cells fully permissive for CMV infection *in vitro* are human fibroblasts, with the CMV replication cycle slower than that of the α-herpesviruses. CMV is, among the herpesvirus family, the single greatest cause of morbidity and mortality in the immunocompromised patient. As with all the herpesviruses described previously it is a widespread virus in most populations with over 50% of individuals commonly infected by adulthood (reviewed in Ho, 1990). CMV may be transmitted casually via infected saliva or sexually via semen or cervical secretions (Wenzel *et al.*, 1973 and Lang *et al.*, 1974). Sexual transmission of CMV is particularly common in homosexual males (Spector, *et al.*, 1984). Maternal-infant transmission has also been noted *in utero* and via infected breast milk (Dworsky *et al.*, 1983). Primary infection, usually early in life, is predominantly sub-clinical although if transmission occurs *in utero,* especially in the first trimester of pregnancy following maternal primary infection, the foetus may experience cytomegalic inclusion disease, a syndrome that can result in the subsequent still-birth of the child or damage to a number of foetal organs such as the CNS, liver and kidneys (Sawyer *et al.*, 1987).

After primary infection CMV can be isolated from monocytes and T-lymphocytes, the virus has also been detected in endothelial and ductal epithelial cells in the salivary glands where a chronic low-level production of virus, in a similar manner to EBV, may be established and be a source of transmissible virus to other susceptible individuals. Cytomegalovirus appears to achieve latency in peripheral blood mononuclear cells, with approximately 1 in 10^5 of such cells containing viral genome (Garrett *et al.*, 1982). Latency is maintained by the CMV major immediate-early gene (IE1). Reactivation of CMV from latency may occur frequently and as with primary infection is usually sub-clinical, however these periods of reactivation may be prolonged due to the ability of the virus to evade certain parts of the immune system. The virus is able to transfer directly from cell to cell thus reducing its immunological "profile" to the cell-based and humoral arms of the immune system. CMV also encodes a polypeptide (the UL18 protein) which has homology to the heavy chain of the MHC class I molecule. This homologue can bind with circulating cell-free β2-microglobulin causing interference with CD8+ve T-lymphocyte recognition (Browne *et al.*, 1990). Such CD8 T-lymphocyte recognition has been shown, in murine CMV models, to be important in immune control of CMV infection. By these mechanisms persistent low-titre, as well as latent, CMV infections may occur, with up to 10% of infected individuals found to be shedding virus, usually in saliva or semen, at any one time (Roizman, 1993).

Whilst infection in the immunocompetent individual is of little clinical significance CMV replication in the immunocompromised host, usually due to reactivation from latency, may cause severe disease with associated morbidity and mortality. CMV-associated disease is of such concern in the immunocompromised host that great care is taken not to introduce CMV-infected organs or blood products into a CMV naive solid-organ transplant recipient or to introduce CMV-naive bone marrow into a previously CMV-infected bone marrow recipient (see section 3. d.). However even if transmission of CMV at the time of transplant can be avoided the level of

immune suppression associated with such transplantation is sufficient to allow reactivation of CMV in a previously infected host. Up to 50% of CMV seropositive transplant patients may experience CMV-associated disease due to viral reactivation (Pollard *et al.*, 1988).

In the transplant patient population CMV disease usually presents as a high grade fever, unresponsive to antibiotics, one to four months post-transplant. This fever is probably caused by the systemic spread and replication of the virus throughout the body. The virus may then cause specific disease at a number of organ sites including the kidneys, liver and, most severely in bone marrow recipients, the lungs (Weiner *et al.*, 1986). Such a pneumonitis, even with appropriate CMV therapy, has an associated mortality rate of approximately 50%. If untreated, or if treatment is delayed, over 90% of affected patients will die of respiratory failure (van der Meer *et al.*, 1996). Primary CMV infection and CMV reactivation have also been associated with organ graft rejection and graft versus host disease (GVHD) in all forms of transplant, especially in those patients receiving renal and heart transplants (Grattan *et al.*, 1989). Because of the high levels of morbidity and mortality experienced from CMV replication in immunosuppressed transplant patients it is common to treat prophylactically all patients, with serological evidence of previous CMV infection, with ACV throughout their period of immunosuppression. (Wolf *et al.*, 1993).

The spectrum of CMV disease in patients who are immune-suppressed due to infection with HIV differs subtly from that seen in the transplant patients. This is due to the more extensive type of immunosuppression that HIV-infected patients experience, especially in their ability to mount a cell-mediated immunological response. CMV disease is also more common in HIV-infected patients who have contracted HIV through homosexual contact, reflecting the increased number of CMV strains these patients have been exposed to through such sexual contact (Drew, 1984). Instead of CMV pneumonitis, which is thought to require host T-lymphocyte activity, as seen in transplant recipients,

the most common sites of CMV disease in HIV-infected patients are the CNS, the eye and the gastrointestinal (GI) tract. Infection of the eye may occur in up to 30% of patients who have contracted HIV by homosexual contact and affects the cells of the retina (Jabs *et al.*, 1989). If left untreated such infection will result in rapid destruction of the retinal surface through direct viral lysis and irreversible blindness. Following an initial unilateral retinal CMV infection subsequent involvement of the second eye occurs in approximately 40% of patients (Jacobson *et al.*, 1988). Infection of the GI tract may result in a chronic disease characterised by mucosal ulceration, profuse watery diarrhea and abdominal tenderness (Meiselman *et al.*, 1985). Although not directly life-threatening such disease is thought to contribute to the syndrome of "HIV-wasting" in which patients may lose up to 35% of their body weight and have an associated poor life expectancy. With greatest significance to patient health is CMV infection of the CNS. CMV encephalopathy and polyradiculopathy are both common end-stage diseases in AIDS and have a high associated mortality. The proportion of HIV-infected patients that die as a direct consequence of CMV disease is difficult to establish accurately. By this late stage of AIDS, patients are commonly suffering from multiple organ disease caused by a wide range of opportunistic infections and malignancies. However some post-mortem studies of gay men who have died of AIDS have shown that nearly half have direct damage of the CNS associated with CMV infection (Morgello *et al.*, 1987).

As well as behaving as an opportunistic pathogen in patients with HIV-infection there is a significant body of evidence that shows CMV infection directly contributing to the immune destruction these patients experience. Indeed primary CMV infection in homosexual men may in itself lead to an inversion of the CD4+ve / CD8+ve lymphocyte ratio which is a hallmark characteristic of AIDS (Carney *et al.*, 1981). CMV, like HHV6 and EBV, may also destroy a number of cells important in control of the immune system by infection of lymphoblasts, granulocytes and mononuclear cells (Detels *et al.*, 1984). It also interferes with certain leucocyte functions including the release and response

to Il-1 and Il-2 by T-cells, HLA-DR expression and antigen presentation by monocytes (Kapasi *et al.*, 1988). In addition to these direct effects CMV has been found to interact with HIV-1. *In vitro* studies have found that lymphoblastoid cell cultures infected with both CMV and HIV-1 show complete cellular lysis, whereas HIV-1, or CMV-only infected cells show only 30% lysis (Casareale *et al.*, 1989). Bi-directional enhancement of CMV by HIV-1 and HIV-1 by CMV has been observed in CD4+ve cell lines, the later due to the transactivation of transcription by CMV gene products of the long terminal repeat sequence of HIV-1. Such interaction *in vivo* has been suggested to be important in the progression of HIV-infection leading to the development of the AIDS complex (Duclos *et al.*, 1989 and Wiley *et al.*, 1988). Additionally HIV-1 infection of monocytes has been shown to render these cells permissive for CMV infection and subsequent viral lysis. Analysis of seroepidemiological data suggests that HIV-1 infected patients with active CMV infection are indeed at a greater risk of developing AIDS than CMV-uninfected patients and that CMV retinitis is associated with HIV-1 p24 antigenemia and severe immune deficiency, whereas HIV-1 p24 antigenemia is by itself not associated with a CD4 count decrease (Fiala *et al.*, 1986).

CMV disease in the immunocompromised host can be seen to be of extreme clinical importance. Since the number of patients undergoing organ transplantation is increasing and patients with HIV-infection are living longer CMV-associated morbidity is likely to become evermore burdensome. Unlike HSV-1 and 2 and VZV, ACV has little effect against active CMV disease, although it appears a reasonable prophylaxis treatment in preventing CMV disease in organ transplant recipients (Meyers *et al.*, 1988). There are however two highly effective drugs, ganciclovir (GCV) and phosphonoformic acid (foscarnet, PFA) which are currently licensed for treatment of CMV disease (reviewed by Serody, 1993). Both GCV, a nucleoside analogue, and PFA, a pyrophosphate analogue can interact with the CMV-encoded DNA polymerase enzyme in preference to the host-cell polymerase thereby inhibiting synthesis of new viral genomes. GCV may also function in the triphosphate

modification as a chain terminating molecule in a similar manner as ACV-triphosphate. Unlike ACV, both GCV and PFA are highly toxic *in vivo* causing severe neutropenia and thrombocytopenia, and nephrotoxicity respectively (Buhles *et al.*, 1988 and Meyers *et al.*, 1988). This toxicity makes prophylactic use inappropriate and even when restricted to use in severe and life threatening CMV disease either drug may have to be withdrawn or exchanged for the other. Recently there have also been an increasing number of reports of CMV isolates which have phenotypic resistance to one or both drugs, either by mutation in the their CMV polymerase gene product or in the CMV UL97 gene product which is a thymidine kinase-like molecule responsible for the initial phosphorylation of GCV to GCV-triphosphate (Drew *et al.*, 1991, Baldanti *et al.*, 1995 and Chou *et al.*, 1997).

The development of improved diagnostic techniques for the diagnosis of CMV disease in the immunocompromised host and the monitoring of response to treatment in a variety of affected patient groups forms a major section of this thesis. The monitoring of systemic CMV DNA levels in recipients of allogeneic bone marrow transplants is described in section 3. d.. This study was undertaken in an attempt to reduce the time required for chemotheraputic intervention in patients at risk of CMV pneumonitis and investigate the incidence of active CMV replication in a cohort of these severely immunocompromised patients in the first six months after their transplant. The association between ocular and neurological CMV infection and disease in end-stage AIDS patients is also examined in section 3. e..

1. a . 7. Human herpesviruses 6 and 7

Both these β-herpesviruses have only recently been described and are now known to be closely related at the genomic level (Salahuddin *et al.*, 1986 and Frenkel *et al.*, 1990). They were each first isolated from the peripheral blood of HIV-infected individuals and can be cultured to high titre in T-lymphocyte

cultures, reflecting their *in vivo* sites of replication. Although common at extremely high frequency world-wide, due to their apparent ease of casual transmission via infected saliva, neither human herpesvirus 6 (HHV6) nor 7 (HHV7) are associated with significant disease in the immunocompetent host. However soon after discovery it was shown by *in vitro* studies that HHV6 was able to transactivate the HIV long terminal repeat (LTR) sequences involved in initiation of HIV replication and that both HHV6 and HHV7 could co-infect the same target cell as HIV; the CD4+ve T-lymphocyte (Gallo *et al.*, 1990). It was therefore suggested that HHV6 infection, or reactivation, may act as a co-factor for the up-regulation of HIV in infected individuals and may be associated with progression of HIV-infected patients to the AIDS syndrome. Currently little epidemiological evidence exists to support this hypothesis and although single case studies have found HHV6 to be present in a variety of HIV-associated systemic tumors and CNS disease there is only rare evidence for significant disease causation by either these viruses even in highly immunosuppressed individuals (Fox *et al.*, 1988). The presence of HHV6 and HHV7 DNA in ocular fluid and neurological tissue of individuals who died of AIDS-related disorders is investigated in section 3. f. This analysis was performed to determine whether either of these viruses may be associated with disease pathology at these specific sites in patients with extremely severe immunodeficiency.

1. a. 8. Human herpesvirus 8 / Kaposi's sarcoma-associated herpesvirus

Human herpesvirus 8 (HHV8) is the most recently described of the *herpesviridae* and like EBV belongs to the B-cell associated γ-herpesvirus group. Unlike the previous seven human herpesviruses HHV8 was not discovered by culture isolation from clinical material but by direct detection of sections of its genome sequence using the method representational difference analysis (RDA) in tissue samples from an HIV-infected patient with the tumour Kapsoi's sarcoma (KS) (Chang *et al.*, 1994). The possibility of an

infectious cause for KS was first suggested over thirty years ago (Giraldo *et al.*, 1972), but it was not until RDA was performed and HHV8 DNA discovered in KS tissue that a probable link with any one pathogen was proposed.

KS is characterised as an angioproliferative, multifocal neoplasm localised mainly in the skin, but which may have systemic involvement especially of the lungs, oral cavity and GI tract (Friedman-Kein, 1982). Macroscopically the cutaneous lesions first appear as small, flat lesions which progress to raised purple nodules. These are generally painless, non-puritic and follow the sub-dural pattern of lymphatic drainage. As the lesions develop they may be associated with local edema and can evolve to form large infiltrating lesions with or without ulceration and secondary bacterial infection (reviewed by Levine *et al.*, 1992). Histologically all forms of KS (see below) are indistinguishable. The tumour is highly vascular, consisting of open slits of endothelial spindle cells, lymphatic channels, an inflammatory infiltrate and extravasculated erythrocytes. The spindle cells secrete paracrine factors and cytokines that appear to promote angiogenesis (Ensoli *et al.*, 1989). The mononuclear cells secrete cytokines which in turn stimulate the growth of the spindle cells (Barillari *et al.*, 1992), these appear to be obligatory for the formation of the tumour. *In vitro* studies have shown that the spindle cells express receptors for a number of cytokines, including Il-1, Il-2, Il-6 and Il-8 as well as TNF, they also themselves produce high levels of Il-1 and Il-6 (Miles, 1993). The cell origin of KS spindle cells remains uncertain but it is probably a mesenchymal progenitor of endothelial lineage (Uccini *et al.*, 1994). It is still unclear whether KS can be regarded as a true malignancy or is instead a multicentric proliferation of abnormal endothelial cells (Miles *et al.*, 1993).

Before the HIV-pandemic KS was largely restricted to the members of particular populations such as elderly southern Mediterranean males (classical KS) and children and adolescents from sub-Saharan Africa (endemic KS).

Patients who received immunosuppressive therapy for organ transplant operations also had an increased incidence of KS (iatrogenic KS) (Jacobson *et al.*, 1995). Outside these groups the annual incidence of KS in the UK and USA was extremely low, at approximately 0.02 - 0.06 per 100,000 of the population per year (Bigger *et al.*, 1984). In 1981 there were a number of reports of previously healthy young gay men in New York and San Francisco presenting with an aggressive type of KS (Centre for Disease Control, 1981). None of these men had any of the previously defined risk factors for the disease, although all were found to have inverted ratios of blood CD4+ve and CD8+ve T lymphocytes. The emergence of KS in this group (now termed epidemic, or AIDS-KS) was amongst the first clinical evidence of the start of the HIV epidemic. In the HIV-infected gay population the incidence of KS has now remained fairly stable over the last fifteen years with about 30% of HIV-infected gay men suffering from KS during the course of their HIV infection (Jacobson *et al.*, 1990 and Peters *et al.*, 1991). However the overall patient mortality from KS has increased during this period, probably due to effective treatment of other opportunistic pathogen diseases such as *Pneumocystis carnii* pneumonia (PCP). Studies have shown that KS, mostly because of pulmonary involvement, may now be responsible for over 30% of HIV-infected homosexual deaths and can be found systemically in over 50% at post-mortem (Ndimbe *et al.*, 1994). In contrast the tumour remains extremely rare in HIV-infected patients who do not originate from sub-Saharan Africa or who do not have homosexual contact as their prime risk factor for HIV-acquisition (Beral *et al.*, 1990).

Before the discovery of the HHV8 sequences a number of viral pathogens had been suggested as possible causative agents of KS, these included hepatitis B virus, CMV, human papilloma virus and HHV6, indeed viral particles of a herpesvirus morphology had been visualised in KS lesions as early as 1972 (Giraldo *et al.*, 1972). However the prevalence of these viruses did not accurately match the incidence of KS, nor the specific geographical, racial and sexual-preference backgrounds of individuals at high risk of KS development.

If a herpesvirus, in particular, was to be the cause of KS it would need to differ in its epidemiological characteristics from all previously discovered human herpesviruses by having a markedly restricted geographical prevalence while being present at high frequency only in those patient groups affected.

Although the abnormal endothelial and epithelial cells may be cultured from KS tissue HHV8 has yet to be isolated directly from such cultures, even though these cultures have subsequently been shown to contain HHV8 DNA (Boshoff *et al.*, 1995a). The failure to isolate infectious particles from KS tissue and cultured cell lines led to the use of the RDA method by Chang and colleagues in 1994. RDA allowed comparison between the genetic compliment of KS cells and enabled isolation of sequence differences between that DNA and the DNA present in unaffected cells (Lisitsyn *et al.*, 1993). This led to the discovery of two small sections of DNA specific to the KS tissue. Identification of these sequences enabled design of oligonucleotide primers for detection of the novel sequences by the polymerase chain reaction (PCR) (see section 1. b. 3.) and allowed comparison with previously described viral genome sequences. When compared with known herpesviral genomes the sequences were found to have a high degree of homology with the γ-herpesvirus group, especially HVS and EBV. The virus from which these sequences were thought to be derived was provisionally called Kaposi's sarcoma-associated herpesvirus (KSHV), it is now also known as human herpesvirus 8 (HHV8). Once PCR reagents were available for the detection of HHV8 DNA sequences a number of studies were conducted to determine their prevalence in a variety of tissues. It was found that almost all HIV-associated KS tumors contained HHV8 DNA, but in only very few non-KS control skin samples could the virus be detected (Boshoff *et al.*, 1995b, Moore *et al.*, 1995, Schalling *et al.*, 1995, and Dupin *et al.*, 1995). Viral particles with typical herpesviral morphology have now been visualised by electron microscopy from HHV8 DNA positive KS skin lesions (Orenstein *et al.*, 1996). HHV8 DNA was also found at other sites in the body, in the blood of HIV-infected patients with KS(Whitby *et al.*, 1995), as well as bronchial

washings from HIV-infected patients with the pulmonary form of KS (Howard *et al.*, 1995). In the non-HIV infected population HHV8 DNA was not found in a large variety of cutaneous cancers but was consistently found in KS tumours from the risk groups described above. These initial findings supported the theory of a causal association between HHV8 and KS. As well as being associated with KS, HHV8 DNA was also detected in a number of other, predominantly HIV-associated, malignancies including the rare body-cavity based lymphomas (BCBL) and multicentric Castleman's disease (MCD, Cesarman *et al.*, 1995a and Soulier *et al.*, 1995). Recently an association with multiple myeloma has been suggested, however to date there has been little independent confirmation of this finding (Rettig *et al.*, 1997a, see section 3c,).

HHV8 has now been grown in a continuous culture system using HHV8-transformed B-lymphocytes from patients with BCBL (Arvanitakis *et al.*, 1996 and Boshoff *et al.*, 1998). In these cell lines (see section 2. a.) HHV8 is latently present in all cells, with or without the additional presence of EBV, at a concentration of approximately 20 - 100 copies per cell. In this latent state HHV8 produces a sufficient antigenic profile to have enabled use of the cell lines in the development of serological assays for the detection of antibodies to HHV8 (Miller *et al.*, 1996, Gao *et al.*, 1996 and Simpson *et al.*, 1996). Such assays are analogous with those developed for the detection of EBV antibodies using EBV latently-infected lymphoblastoid cell lines (Hotchin *et al.*, 1991). Active expression of the viral lytic-cycle proteins can also be achieved in these cell lines by stimulation with a number of compounds, including sodium butyrate (Lennette *et al.*, 1996). To date no HHV8-susceptible cell lines have been described although there have been reports of the adenoviral-transformed cell line 293 supporting some viral replication (Foreman *et al.*, 1997).

Indirect immunofluorescence (IF) assays, using both the HHV8-infected BCBL cell lines, and recombinant HHV8 proteins have allowed some epidemiological surveys of the prevalence of HHV8 (see sections 2. d. and 2.

e.). These studies indicated that, as expected, the virus is present in a very low percentage of western populations; the seroprevalence in the UK and USA would appear to be between approximately 1 and 5% (Simpson *et al.*, 1996). In populations from areas where KS is endemic the seroprevalence is between 35-70% (Gao *et al.*, 1996, Kedes *et al.*, 1996, Miller *et al.*, 1996 and Chang *et al.*, 1996). Antibodies to the virus are also present in a high proportion of gay men and in nearly all HIV-infected gay men with KS (Kedes *et al.*, 1996 and Chang *et al.*, 1996). Currently almost all patients with classical KS have been found to have detectable circulating levels of antibodies to HHV8 (Simpson *et al.*, 1996).

It has also been found that detection of the viral genome and antibodies in the peripheral blood of HIV-infected patients without KS is highly predictive of subsequent KS development, further underlining the probability that HHV8 infection is required for KS development (Whitby *et al.*, 1995, Moore *et al.*, 1996 Gao *et al.*, 1996 and Martin *et al.*, 1998). The very low levels of seroprevalence in the general population, far lower than for any other human herpesvirus, may reflect a number of important features of the virus. Transmission appears to be predominantly by intimate sexual contact especially of a homosexual nature. A recent study has linked type and frequency of homosexual sex with the incidence of HHV8 infection (Martin *et al.*, 1998). Also the virus may be classed as an "emerging virus", as has been suggested for HIV, and that only recently has HHV8 spread from its established endemic areas to infect certain individuals, such as homosexual men, in other populations (Zhong *et al.*, 1997).

The mechanisms by which HHV8 may actually cause disease are still far from well understood. The virus has a large linear genome of double-stranded DNA about 170 Kb in size. The central 140 Kb of the genome are unique and have a low G / C content of approximately 54% (the L region), flanking this unique sequence are tandemly repeated 800 nucleotide repeats which contain a much higher G / C content of over 80% (the H region). The full genome sequence

has now been determined from both a BCBL cell line virion and a clinical isolate (Russo *et al.*, 1996). HHV8 contains over 70 conserved herpesviral genes with significant homology to the gene blocks of the New World primate tumour herpesvirus HVS, and to EBV, and its genomic organisation mirrors that found in these two closely related viruses (Albrect *et al.*, 1992 and Moore *et al.* 1996).

In common with other γ-herpesviruses the HHV8 genome encodes for a number of protein homologues to previously described human cell cycle regulation proteins. One such homologue (HHV8 ORF 16) is analogous to the cellular apoptosis-inhibiting protein Bcl-2 (Chang *et al.*, 1996b). Cellular Bcl-2 can bind to the Bax family of proteins *in vivo* preventing cell death, a common host response to viral infections (Sato *et al.*, 1994). By encoding this analogue the virus may be able to prolong the life-span of HHV8-infected cells in order to support greater genome replication and escape the effects of the host immune response. A number of human cancers, including B-cell malignancies (Gauwerky *et al.*, 1988) have been found associated with a disregulation of cellular Bcl-2 production, probably through its anti-apoptotic effect (reviewed by Reed, 1995). HHV8 also encodes for a homologue to the cellular type-D cyclin (Cesarman *et al.*, 1996 and Chang *et al.*, 1996b). This viral cyclin (v-cyclin) displays a 31% homology with cellular cyclin D2 (Li *et al.*, 1997) and transcripts for its production have been found in the majority of HHV8-infected spindle cells (Davis *et al.*, 1997). Cellular D-type cyclins are part of the family of cellular cyclins which control cell cycle and genome replication (reviewed in Sherr, 1996). The cyclins function by binding to cyclin-dependent kinases (cdks) which in turn phosphorylate other proteins involved in cell cycle regulation. Type-D cyclins act during the mid-to-late G1 phase of the cell cycle by complexing with cdk-4 or cdk-6 to phosphorylate the retinoblastoma tumour suppressor protein (pRb) which controls cell entry into the S phase of division. Unphosphorylated pRb blocks cells in the G1 phase by binding to a number of proteins including a family of transcription factors known as E2F. However the phosphorylation of pRb by D-cyclin

causes the release of E2F which is necessary for cellular genome replication and hence cell division. Expression of the viral D-cyclin therefore may lead to cell cycle disregulation by causing the unregulated phosphorylation of pRb. The importance of the pRb tumour suppresser pathway to control cell cycle progression is underlined by the fact that both the other well studied tumour-forming γ-herpesviruses, EBV and HVS, cause pRb phosphorylation thereby promoting neoplastic transformation of the infected cells. HVS, like HHV8 encodes a functional human cyclin homologue (Nicholas *et al.*, 1992), whereas EBV induces over-activity of the cellular cyclins in infected cells (Sinclair *et al.*, 1995).

Another mechanism by which HHV8 infection may result in uncontrolled cell replication is by production of a viral homologue (v-Il-6) to cellular Il-6 (Neipel, 1997). Il-6 was originally termed the B-cell growth factor and is an essential cytokine required for the differentiation of B lymphocytes, the establishment of B cell-derived lymphomas, and the proliferation of KS-derived cell lines (Scala *et al.*, 1994). The unregulated production of v-Il-6 in BCBL's and KS is therefore highly likely to contribute to their malignant growth, especially as KS spindle cells have been found *in vivo* to express a high-affinity IL-6 receptor. Importantly v-Il-6 has already been found to support growth of cellular Il-6-dependant cell lines so functional activity *in vivo* is probable (Moore *et al.*, 1996).

Although EBV is the closest human relative of HHV8 as yet no HHV8 homologues to the EBV EBNA's or LMP's have been identified. In EBV-infected cells these proteins have been shown crucial for the maintenance of viral latency and host cell transformation (see section 1. b.). However although lacking in these gene homologues HHV8 has a number of open reading frames which may express proteins homologous to the cellular gene products which are themselves induced by EBV through the activity of the EBNA and LMP products; As well as cyclin D, Il-6 and Bcl-2, the functions of which are described above, HHV8 also encodes homologues to the complement

controlling protein CR-2 and an Il-8 receptor which may have a role in angiogenesis, a feature fundamental to the development of KS tumours (Birkenback *et al.*, 1993 and Burgstahler *et al.*, 1995). It would appear that in contrast to EBV, which produces viral proteins capable of interacting with host cell proteins which then cause disruption of the normal regulation of cell cycle control, HHV8 may itself code directly for replicas of the host anti-apoptotic and regulation genes, and it is these viral proteins which directly promote the immortalisation of infected cells (reviewed in Neipel *et al.*, 1997b and Chang, 1997) as depicted below:

EBV gene/protein	**Cell protein induced**	**Effect**	**HHV8 gene/protein**
EBNA 2 / EBNA LP	cyclin-D	cell cycle control	v-cyclin
LMP-1	bcl-2	anti-apoptosis	v-bcl-2
gp350 / 220	Il-6	growth factor	v-Il-6

With the extremely complex mechanisms of angiogenesis and cell transformation involved in the formation of a KS lesion it does however remain probable that HHV8 is not the sole required entity for KS lesion development. Instead a two- or three factor model involving host cell chemokine molecules such as MIP-1α, the HHV8 gene products and possibly even interactions with HIV *tat* protein have also been suggested (Neipel et al., 1997b). The state of the host immune system would also appear critical in allowing disease formation, a factor well described in association with herpesviral pathology and intimately associated with control of herpesvirus replication and dissemination

In summary the association between HHV8 and KS / BCBL appears overwhelming with virus genome present in virtually all KS and BCBL tumours. At the cellular level the virus encodes a large array of gene products able to initiate and support cellular proliferation and these products have been shown to have parallels in the other known transforming herpesviruses.

This thesis describes early work relating to the role of HHV8 in KS. The work was started a few months after the first HHV8 publication by Chang and colleagues (Chang *et al.*, 1994) and includes studies describing viral prevalence in a range of individuals. Subsequently it has been possible to develop assays for the detection of HHV8 which have been used in the diagnosis of different forms of KS. The effect of a number of chemotheraputics on systemic and localised HHV8 DNA titre and KS clinical severity has also been investigated. More recently possible mechanisms of HHV8 transmission have been studied, further defining the epidemiology of the virus. These studies have made an important contribution to the understanding of the role that HHV8 plays in KS disease and in determining the prevalence of this new virus in a variety of patient populations.

1. b. Virological Diagnostic Techniques

The following section describes the assays used in this thesis and discusses the more recent technology in the context of the established assay types.

To make the diagnosis of a viral infection the virus itself and/or the immunological response of the host to that virus may be detected. Each of the assays described provides differing information and each has advantages and limitations which must be considered. Both the type and quality of clinical sample available, as well as knowledge of the biological properties of the viral type to be investigated are vital in the selection of an appropriate diagnostic technique. In general however there are four features of any diagnostic test which must be evaluated to determine suitability in each situation:

1. The *sensitivity* of the assay; this is the percentage of individuals who are truly infected which can be detected by the assay.

2. The *specificity* of the test; this is the percentage of individuals who are not infected but in whom the assay still makes a detection.

3. The *diagnostic window*; the time between infection with an agent and the point at which an assay can first detect that infection.

4. The *operational considerations*, the price of the assay, both financially and in terms of the intensity of laboratory work required to carry out that assay.

In terms of the diagnosis of herpesvirus infections a large number of techniques are currently available. These range from those which have been in use for over 30 years, such as viral culture and direct electronmicroscopy, to those developed in the last few years including the latest genome detection methods. Very rarely is one type of test used in isolation, but usually a range

of tests are employed to provide maximum clinical information in the context of the first three points outlined above.

1. b. 1. Virus detection

The oldest method of identifying a viral species is by it's growth. Originally this was performed in embryonated eggs, now it is usually in cell culture preparations. In cell culture a specimen is added to a susceptible cell-line capable of supporting viral replication and if infectious virus is present then the virus will grow and produce a change in the morphology of the cell line. This is called a cytopathic effect (CPE). The CPE can be characterised microscopically and the virus type determined. Virus isolation has long been the bench-mark for identification of virus infection and characterisation. A variety of clinical samples may be investigated by inoculation into cell culture. Samples such as vesicle fluid and skin scrapings can be recovered from the site of disease for determination of a specific site diagnosis. Excreted fluids such as urine and saliva may be examined for evidence of systemic infection and blood can be investigated for the presence of viraemia. Table 1. 2. shows the various cell lines used in culturing herpesviruses and the other methods employed for herpesviral identification.

Although cell culture techniques are still widely used in all diagnostic laboratories they suffer from a lack of sensitivity in comparison to some of the more modern methodologies. For a definitive diagnosis to be made the cultures may have to maintained for up to four weeks prior to the development of CPE. In an effort to increase the sensitivity of virus isolation and to reduce the amount of time required between specimen inoculation and assay result antigen detection techniques have been combined with conventional cell-culture (Gleaves *et al.,* 1984). In simple virus isolation virus detection is only possible when a CPE becomes grossly visible, however prior to this virus replication is occurring in the cells with the expression of a variety of virus-encoded proteins. Antibodies to these viral proteins are available and can be

Table 1. 2. Commonly used assay systems for detecting herpesviruses

Herpesvirus	Cell culture		Antigen detection	Genome detection (e.g. PCR)
	Culture	Identification		
HSV-1	HEL	CPE	EM / direct IF	rarely *
HSV-2	HEL	CPE	EM / direct IF	rarely *
VZV	HEL	CPE	EM / direct IF	rarely *
EBV	none	-	none	yes
CMV	HEL	CPE / DEAFF	pp65 antigenemia	yes
HHV-6	T cell lines	CPE / IF	none	yes
HHV-7	T cell lines	CPE / IF	none	rarely
HHV-8	none	-	none	yes

* - except in cases of suspected viral encephalitis

HEL - human embryonic lung cells

CPE - cytopathic effect

DEAFF - detection of early antigen fluorescent foci

IF - immuno-fluorescence

EM - electron microscopy

PCR - polymerase chain reaction

used to examine the cultures for these viral antigens. Visualisation of bound antibody is by a revealing agent, usually either a fluorescent or enzymatic moiety attached to the antibody. Such techniques, termed immunofluorescence assays (IFA), or enzyme immuno-assays (EIA) can reduce the culture time necessary for viral identification from a number of weeks to 24 hours. Use of these antibody techniques also increases the specificity of the test compared with culture alone. A comparison between viral culture and the DEAFF test, which is based upon the antibody detection of viral antigens revealed by a fluorescent signal, for the detection of CMV infection is described in section 3. d.. The more modern technique of genome analysis is included in this analysis.

1. b. 2. Antibody measurement

Serological assays for the identification of a patient's antibody response to a viral infection are the alternative diagnostic methods to actually detecting the virus, or parts of its structure, as described above. The production of anti-herpesvirus IgG antibody is considered diagnostic of a primary infection, although the IgG titre may also rise during viral reactivation and re-infection. The other class of antibody produced in primary infection is IgM. Immunofluorescence (IF) assays have been widely used for the detection of IgG and IgM response to herpesvirus infections especially in the diagnosis of γ-herpesvirus infections such as EBV and HHV8 (Liaburf *et al.,* 1975, Hotchin and Crawford, 1990, Gao *et al.,* 1996 and Simpson *et al.,* 1996). In these assays infected cells are used as a source of antigen, onto which patient sera is incubated. If antibodies to the viral antigens are present in the infected cells then they will bind and can be visualised as for the IFA described above. There is however a potential for serological cross-reaction with the use of IF assays, particularly between viruses in the same sub-family (Cradock-Watson *et al.,* 1979). For this reason indirect solid-phase immunoassays, such as the enzyme-linked immunosorbant assay (ELISA) have been developed and used for the detection of anti-VZV, HSV and CMV antibodies (described and

discussed in Longston, 1990). In such an assay viral antigen is fixed to a solid phase and is incubated with the patient's sera. After washing away unbound IgG's the virus-specific antibody is detected using an enzyme-labeled anti-human IgG conjugate with a substrate specific for the enzyme.

The development of serological assays for detection of antibodies to HHV8 has been invaluable in defining the association of the virus with disease in both the immunocompromised and immunocompetant host. In this thesis both the IF and ELISA formats of assay have been used to detect anti-HHV8 antibodies in patient groups as described in section 2. d. and 2. e. The use of these assays is detailed in section 3. c..

1. b. 3. Genome detection

A recent advance in the diagnosis of viral infections has been the development of the polymerase chain reaction (PCR). This technique allows the specific amplification of parts of the viral genome in a clinical specimen without need for prior culture in permissive cell lines (Saiki *et al.*, 1988). These amplified genome sections may then be easily visualised by a variety of laboratory techniques. Such direct amplification and detection makes viral diagnosis by this method rapid and because of the processes involved enables single viral genomes present in a clinical sample to be identified (Simmonds *et al.*, 1990).

1. b. 3. i) development of a PCR reaction

To design a PCR test for the detection of a particular viral species all that is required is knowledge of a small, unique, section of the viral genome, as little as 200 nucleotide bases is sufficient. To the viral sequences two complimentary oligonucleotides are designed, one for each strand of genome. These synthetic oligonucleotides prime the subsequent exponential amplification of the target sequence using a thermostable DNA polymerase

enzyme. The design of the primers is crucial to the efficient amplification of the viral genome target sequence and their design defines the annealing temperature of the reaction. This is the temperature chosen to allow the most efficient binding of the primer pair to the genome in preparation for the amplification reaction. For the diagnostic detection of viral genomes, where short DNA sections of less than 500 base pairs are amplified, primers between 18 and 24 bases are usually chosen. Amplification of short regions of DNA is in general more efficient than amplification of longer sections of DNA. Primers shorter than 18 base pairs have an increased risk of binding to heterologous target sequences resulting in non-specific DNA amplification, while those longer may require elevated temperatures to dissociate from the their annealing sites. This elevated temperature reduces the activity of the polymerase enzyme and thus effects overall amplification efficiency. Together with the length of the primer the concentration of guanine and cytosine residues (the G / C content) is also crucial and directly affects the annealing temperature by introducing a greater or lesser number of hydrogen-bonding events. Amplification of a section of genome consisting of between a 40 and 60% G / C content is optimal. As well as overall G / C content stretches of repeated nucleotides are avoided to ensure primer annealing and dissociation from the genome is uniform. For most diagnostic PCR assays of the type described above primer pairs will have an annealing temperature between 50°C - 60°C. To calculate the required annealing temperature for any primer pair the following equation can be used to first calculate the primer melting temperature (Tm) (Sambrook *et al.*, 1989):

Tm= 4 x (number of G + C bases) + 2 x (number of A + T bases) °C.

The annealing temperature selected in the PCR will then be approximately 5 °C below the primer Tm's, however empirical analysis may be required to confirm the efficiency of the reaction.

Also critical in the design of primer sequences is the avoidance of base-pairing complementary 3' terminal ends, e.g. -GC 3' and -CG 3'. If this pairing occurs during the amplification process reagents, such as the nucleotides and the polymerase, will be sequestered to the production of primer dimers; short sections of DNA whose sequence is that of the two hybridised primers. Production of primer dimers results in a dramatic reduction in amplification efficiency of the intended target genome sequence and subsequent loss of PCR assay sensitivity.

As well as the design of the oligonucleotide primers the composition of the PCR reaction buffer is important in optimising the amplification reaction. The PCR buffer used for all work in this thesis has, for example, a composition of 50 mM Tris and 50mM KCl dissolved in aqueous solution, a combination well established for use in PCR (Mullis, 1994). The major variable component in the buffer is the concentration of Mg^{2+} ions. Magnesium ions are co-factors for efficient polymerase enzyme activity and throughout the studies described a concentration of 1.5 mM was used. If however Mg^{2+} concentration is too low then the efficiency of the polymerase will be reduced, if too high then the specificity of the polymerase will be reduced (Saiki *et al.*, 1989).

1. b. 3. ii). PCR cycling conditions

Denaturation: Once viral DNA from a sample has been prepared for PCR analysis (see section 1. b. 3. v.) the first step in the PCR reaction is to separate the double-stranded DNA molecule into its two single strands, thus making available the viral genome nucleotide sequence for annealing with the specific oligonucleotide primers. This is achieved by heating the reaction to between 94 °C and 96 °C.

Priming: To allow the annealing of the oligonucleotide primers to the exposed genome sequence the reaction is cooled to between 50°C - 60°C, depending on primer Tm.

Strand extension: When primer hybridisation is complete the temperature of reaction is increased to 72°C. At this temperature the annealed primers remain attached to the viral genome sequences and allow the thermostable polymerase enzyme to bind to this section of double stranded DNA (comprising single-stranded viral genome and hybridised oligonucleotide primer). The bound polymerase then progresses along the single-stranded viral DNA molecule incorporating complementary free nucleotide molecules, made available in the reaction mix. This extension process completes one round of amplification.

During the first cycle of amplification strand extension extends beyond the point on the viral genome at which the other primer binds. However in subsequent cycles an exponentially increasing number of DNA molecules are produced the size of which are defined by the 5' ends of each primer. The overall number of DNA molecules produced by the PCR amplification reaction can be approximately calculated by the equation:

number of DNA molecules = 2×2^{n}

where n = the number of cycles performed.

Visualisation of PCR product molecules: Agarose gel electrophoresis is commonly used to visualise the millions of amplified DNA molecules produced by the PCR (Sambrook *et al.,* 1989). This methodology involves the separation of differently sized double-stranded DNA molecules in an agarose matrix by the application of an electric charge through the matrix. The DNA molecules are negatively charged and will move to the positively charged end of the gel at a speed inversely proportional to their size. By including the DNA

intrachelating dye ethidium bromide in the gel, bands of amplified DNA can be viewed under ultraviolet light. As the positions of the primers in the target genome are known the expected size of the DNA bands can be calculated. Inclusion of a DNA size marker in the gel allows confirmation that the PCR products seen on the gel are of the predicted size and therefore not composed of non-specific DNA, e.g. primer dimers.

1. b. 3. iii). the nested PCR

To increase the specificity and sensitivity of the PCR technique a small aliquot (usually 1 µl) of the amplified DNA can be transferred to a second reaction tube and submitted to a further number of amplification cycles (Simmonds *et al.*, 1990 and Mitchell *et al.*, 1994). This second tube contains the same reaction buffer, nucleotide solution and polymerase enzyme as the first, but instead of providing the original oligonucleotide primers a second set which bind to a nucleotide sequence internal to the first primer pair are provided. Not only does this second, or nested, set of cycles increase the absolute amount of DNA produced, making gel visualisation easier and increasing overall assay sensitivity but, by including different primers which are specific only for the correctly amplified sequence from the first round of PCR, it produces a highly specific amplification product. This product is the result of four independent primer / viral genome hybridisation reactions, therefore further confirmation of the origin of PCR products, by methods such as sequencing, is rarely necessary.

1. b. 3. iv). multiplex PCR

A considerable saving in the amount of time and money taken to analyse a clinical sample for a variety of viral genome targets can be made by employing a multiplex PCR (mPCR) methodology (Chamberlain *et al.*, 1988 and 1992).

In an mPCR a range of primer pairs specific for a number of nucleotide sequences, each unique to a particular virus, are made available for hybridisation. Careful design of the primer pairs used is critical to effective mPCR. Ideally the sets of primers should all have similar Tm scores and amplify sequences of a similar length. This simplifies the optimisation of the reaction. It may also be necessary to provide an increased concentration of both the polymerase enzyme and nucleotides to facilitate the amplification of multiple target sequences. Multiple PCR can be more prone to the production of non-specific amplified DNA molecules and so a nested format is used with a high annealing temperature. Use of high annealing temperatures favours specific primer / target binding. It may also be necessary to increase the number of cycles used in both rounds of amplification to maintain the sensitivity of the assay in comparison to any single PCR (Mullis, *et al.*, 1994). The evaluation and clinical use of a multiplex PCR assay capable of detecting six different herpesviral genomes with a level of sensitivity and specificity similar to those six PCR's performed separately is described in section 3. f..

1. b. 3. v). sample preparation

Before addition to the PCR reaction the clinical material being analysed will usually have been pre-treated. Such treatment has a number of functions; most important of these is the removal or inactivation of biological e.g. haemoglobin, or non-biological e.g. sodium dodecyl sulphate (SDS), inhibitors of the PCR process (Gelfand and White, 1990). These inhibitors usually affect the thermostable polymerase enzyme, lowering or eliminating its activity and so reducing the yield of amplified DNA produced during cycling, thus affecting overall PCR sensitivity. Other factors, such as high salt concentrations, may also affect the primer annealing reaction. Preparation may also be necessary to release DNA from clinical material such as tissue to allow presentation for amplification in the PCR process. As many of the procedures used for the recovery of DNA from tissue samples are themselves inhibitory of the polymerase enzyme such material may have to undergo

further purification processes, such as phenol / chloroform treatment before PCR analysis. The absolute sensitivity of any PCR assay can also be further improved by using preparation methods which concentrate the DNA extracted from a clinical sample. The physical and chemical procedures used for sample preparation in these studies are detailed in section 2. c.

1. b. 3. vi) PCR contamination

A potentially significant problem with the PCR assay is the sensitivity with which it can detect very small amounts of target sequence. Whilst this is a useful characteristic in the detection of small amounts of viral DNA in clinical samples it also raises the possibility of the generation of false-positive signals. The contamination leading to false-positive amplification is usually derived from the reaction products of previous amplifications, rather than from the original samples. As PCR amplicons are generally small (usually between 100 and 200 base pairs) they are very stable and may persist for long periods increasing the chance of their inadvertent addition to subsequent reactions. Throughout this thesis stringent precautions were adhered to (Kwok and Higuchi, 1989) and multiple negative controls included in each assay to avoid and / or detect the generation of such inappropriate PCR signals.

In conclusion the PCR technique is an extremely sensitive, rapid and specific method of identifying viral genomes present in clinical material. It is able to distinguish between very closely related viral species and differentiate between differing strains of the same virus. It is also able to accurately detect very low numbers of viral genome in a background of differing DNA species. Since its first practical application in 1987 (Mullis and Faloona, 1987) PCR has revolutionised diagnostic microbiology and is now replacing a number of older methodologies, such as culture and electron microscopy, as the assay of choice for many viral diagnostic purposes.

1. c. Aims of the study

This thesis concerns the development and use of a range of diagnostic techniques for the detection of herpesvirus infections in the immunocompromised patient.

Most of the work included involves the determination of the epidemiology and disease associations of the recently discovered herpesvirus HHV8, also known as KSHV. The development of genome detection methodologies for this virus are described, as is their subsequent use to analyse virus presence in a variety of patient and control individuals. These studies were conducted to investigate claims made by a number of international research groups that HHV8 is the causative infectious agent of the tumour Kaposi's sarcoma (KS). The effects of a range of potential chemotherapy for HIV-associated KS were also investigated as are potential routes of horizontal virus transmission.

As well as the investigation of HHV8 epidemiology the association of all the herpesviruses with ocular and neurological disease in HIV-infected patients was examined in an attempt to determine the prevalence of herpesviruses at these sites and evaluate current diagnostic PCR assays with clinical and histological diagnosis of herpesviral involvement. Before this study the range and extent of herpesvirus infections at these sites was not fully realised potentially compromising the clinical response offered upon symptomatic presentation.

Another patient group in UCL Hospitals for whom the clinical management of herpesvirus infections was previously felt to require greater diagnostic support were individuals undergoing allogeneic bone marrow transplantation. A six month study was conducted after replacing the existing CMV surveillance protocol of virus culture and DEAFF analysis in this patient group with a nested PCR methodology. It was hoped that this system would allow a

reduction in the toxicity of current anti-CMV prophylactic support and a more rapid therapeutic response to emerging active CMV infection.

Finally in an attempt to simplify and reduce unnecessary cost in the overall herpesvirus PCR diagnostic service offered in the Department of Virology, UCL Hospitals a multiplex PCR assay was developed allowing simultaneous detection of the six most clinically significant herpesvirus infections in the immunocompromised patient. The evaluation of this assay, capable of detecting HSV-1, HSV-2, VZV, EBV, CMV and HHV8 DNA was undertaken using control genomes and comparison made with each of the individual virus nested PCR assays performed separately. A blinded comparison between the two assay formats using clinical specimens previously analysed by the individual nested PCRs was also performed. This new multiplex assay is now replacing the use of the individual viral PCR assays, within the Department of Virology, UCLMS, thus improving the efficiency of the molecular diagnostic service provided.

Section 2: Materials and Methods

2. a. Tissue culture and *in vitro* growth of HHV8

Two non-adherent cell lines were available for propagation of HHV8. The first, HBL-6, was dually infected with HHV8 and EBV (Cesarman *et al.*, 1995) (fig. 2. 1. a). The second, BCP-1, was infected with HHV8 alone (Boshoff *et al.*, 1998) (fig. 2. 1. b). Both were derived from lymphomatous effusions taken from HIV-infected patients with body cavity-based lymphomas (BCBL's). They were received from the The Institute of Cancer Research, London in RPMI medium supplemented with 10% foetal bovine serum (FBS).

2. a. 1. Growth of the HBL-6 cell line

HBL-6 was propagated in RPMI medium supplemented with 10% FBS (complete RPMI, Appendix A) at 37°C and 5% CO_2 under Category 3 conditions. Cells were counted daily in an improved Neubauer haemacytometer (BDH) and had a viability of over 80% if maintained at approximately 5 x 10^6 cells / ml in 30 ml complete RPMI in 75 cm^2 culture flasks (Nunc). This typically required a 1 in 3 dilution (splitting) of the culture in complete RPMI twice a week and transfer of two 30 ml aliquots of diluted culture to two new 75 cm^2 culture flasks, the third aliquot of 30 ml remained in the original flask. Once 12 culture flasks with 5 x 10^6 cells / ml had been established the cells from two flasks were frozen for long-term storage. Cells were pelleted (400g, 10 minutes) and the pellet washed twice with the original volume RPMI. 5 ml freezing medium (Appendix A) was added to the final pellet and the cells resuspended, aliquoted in 1 ml volumes, and transferred into cryotubes (Nunc) for overnight storage at -70°C before placement in liquid nitrogen. When required for use cells were removed from storage and thawed rapidly in a 37°C water bath. They were washed once in 30 ml RPMI

Figure 2. 1. a. HBL-6 cell culture viewed by phase-contrast light microscopy, (magnification x40)

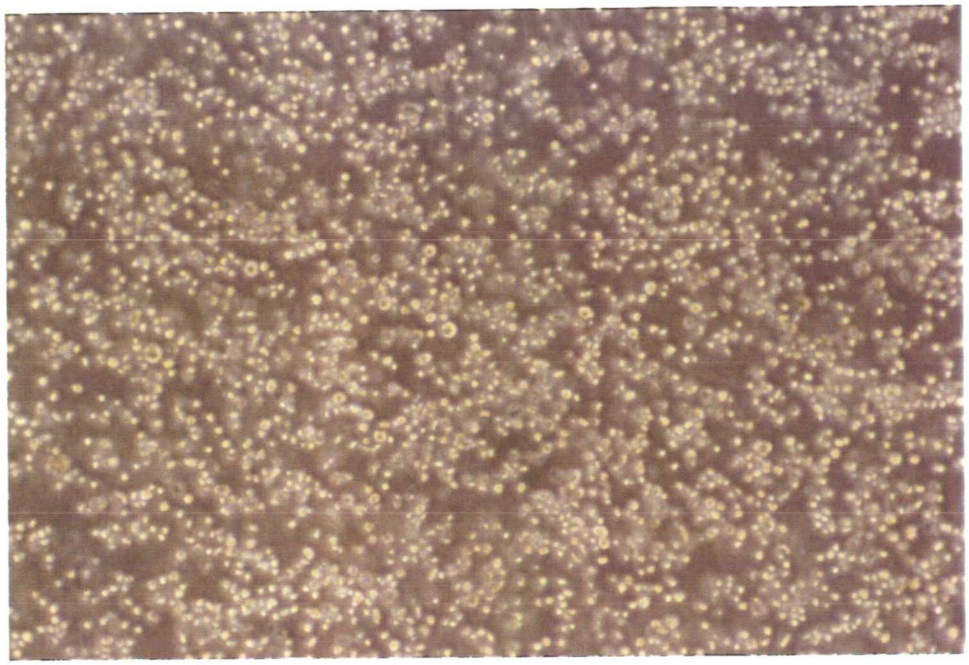
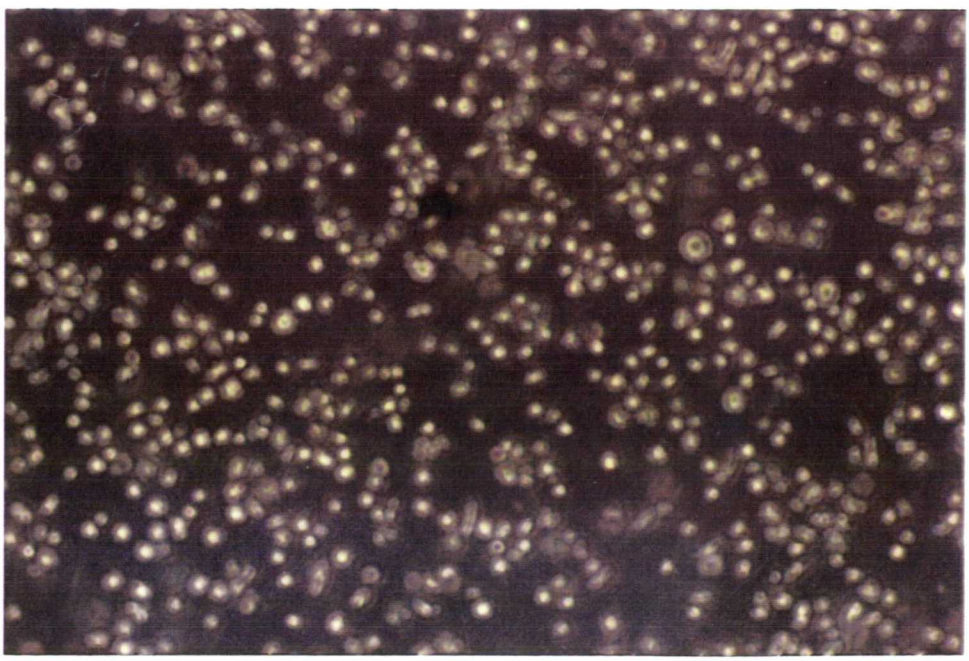


Figure 2. 1. b. BCP-1 cell culture viewed by phase contrast light microscopy. (magnification x100)



and pelleted (400 g for 10 minutes). The cells were then resuspended in 10 ml complete RPMI and placed in a 25 cm^2 culture flask. After three days growth the culture was transferred to a 75 cm^2 culture flask and 20 ml more complete RPMI added. The cultures could then be maintained by splitting as described.

2. a. 2. Growth of the BCP-1 cell line and HEL cells

The second cell line infected with HHV8, BCP-1, was received in the same manner as the HBL-6 line and initially grown in complete RPMI medium with 10% FBS. However it was soon noted that use of the HBL-6 protocol for culture maintenance resulted in a 50% decrease in BCP-1 viability. To increase the viability of this cell line a feeder-cell monolayer of human embryonic lung (HEL) cells was used. HEL cells were revived from liquid nitrogen storage as described for the HBL-6 cultures. After washing in RPMI the HEL cells were resuspended in 20 ml HEL growth medium (HEL GM, Appendix A) and placed in a 75 cm^2 culture flask at 37°C in a 5% CO_2 atmosphere. The flask was incubated resting on its side to provide a maximum surface area for establishment of the HEL monolayer. Visualization by phase contrast light microscopy showed that these fibroblastic cells attached to the plastic of the culture flask after approximately 3 hours and within 24 hours had begun to form a surface-adherent cell monolayer which was confluent 3 - 4 days after inoculation. Once the monolayer had been established the GM was removed and 20 ml HEL maintenance medium (HEL MM, Appendix A) added. The cell monolayers were viable for up to two weeks during which the maintenance medium was changed every 3-4 days. To inoculate new culture flasks with HEL cells an existing monolayer was washed twice in 10 ml phosphate buffered saline (PBS, Appendix A). The monolayer was stripped from the culture flask by addition of 1 ml 1% trypsin solution, followed by incubation at 37°C for 2 minutes. Any remaining monolayer was dislodged from the flask surface by tapping the side of the culture flask firmly. Once the monolayer had been separated from the plastic surface 30 ml of HEL GM was

added, the culture flask was sealed tightly, and the medium agitated within the flask to ensure all the HEL cells were resuspended. To the resuspended cell solution a further 60 ml HEL GM was added and three 30 ml aliquots placed in new 75 cm^2 culture flasks.

To support BCP-1 growth HEL monolayers were prepared to a stage of near monolayer confluence (fig. 2. 2. a.), they were then moved from the Category 2 Biohazard Laboratory in which they were prepared to the Category 3 Biohazard Laboratory. The HEL GM was removed and 30 ml BCP-1 culture at 1-5 x 10^6 cells / ml was added. Growth of the BCP-1 culture in the presence of HEL cell monolayers increased BCP-1 viability to over 90% and allowed maintenance of that viability even when BCP-1 cell concentration approached 1 x 10^7 cells / ml (fig. 2. 2. b). It was also observed that after removal of the BCP-1 culture following growth on the HEL monolayer the majority of dead BCP-1 cells remained attached to the monolayer and so were not transferred to subsequent cultures. Thus BCP-1 / HEL co-cultivation allowed for an enrichment of viable BCP-1 cells after each passage to a new HEL monolayer. After prolonged culture it was noted that growth of the BCP-1 culture on the HEL monolayers was not continuously necessary and that co-cultivation could be alternated with growth of the BCP-1 cells alone. A system of alternate passage growth on monolayers and alone was instigated with no appreciable loss of BCP-1 cell viability. By gradual reduction of FBS concentration in the complete RPMI medium cultivation of BCP-1's in as little as 2% FBS was possible without affecting viability.

The BCP-1 cells were stored frozen as described previously, however use of a freezing medium consisting 50% FBS, 30% DMSO and 20% RPMI was found to increase cell viability upon subsequent culture in comparison with the usual freezing medium. Growth of the BCP-1 cells continuously on HEL monolayers for 3 - 4 passages after revival also increased cell viability and reduced the culture time necessary to increase BCP-1 culture cell concentration to 3 - 6 x 10^6 cell / ml.

Figure 2. 2. a. HEL's grown to near-confluence, viewed by phase-contrast light microscopy (magnification x100)

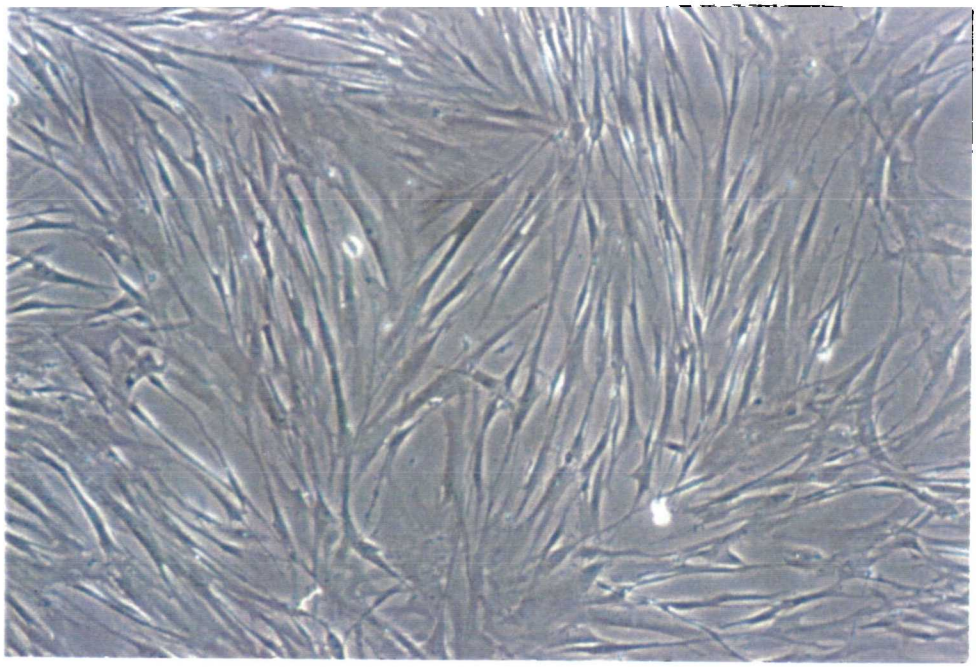
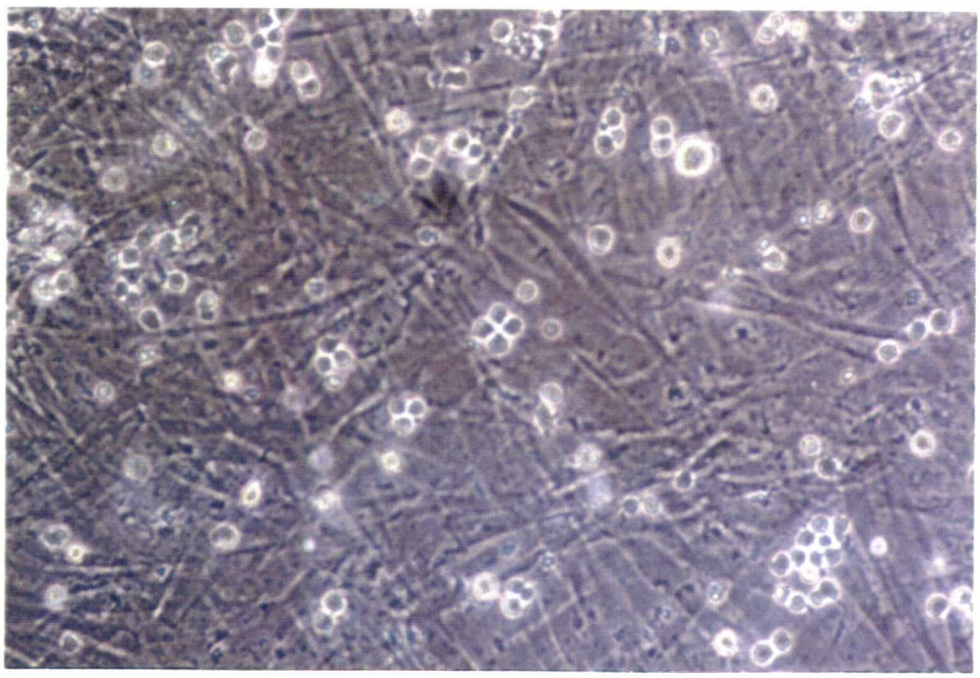


Figure 2. 2. b. BCP-1 cells growing on HEL's, viewed by phase-contrast light microscopy (magnification x100)



2. b. Nested PCR amplifications

This section describes the general protocols for preparing nested PCR assays, the handling of first round amplification products to allow a second round of amplification and the analysis of the resultant DNA molecules (amplicons).

2. b. 1. Preparation of the PCR assay

The first round of the PCR was carried out in a 50µl reaction volume in a 300µl thin-walled microtube (Treff Labs). The reaction mixture contained 1x reaction buffer (from 10x stock, Appendix A), 200µM of each of the four nucleoside triphosphates; dATP, dTTP, dCTP and dGTP (Pharmacia Ltd., supplied as 100mM solutions), 50ng of each outer (first round) primer and 1 unit of recombinant *Taq* polymerase (Applied Biosystems). These reagents were handled using pipettes dedicated for the purpose and 40µl of the reaction mix was aliquoted into each reaction tube. Sample DNA, up to a maximum of 10 µl, was added to all assay tubes except the last 7. In 3 of these last 7 tubes positive control DNA was added, usually at a level of 10 copies, 1 copy and <1 copy respectively. The sample and control DNA was prepared and added using a separate set of pipettes fitted with filter-containing tips to avoid cross-contamination with sample DNA (Kwok and Higuchi, 1989). In the last 4 tubes 10µl of water were added, to 2 tubes with the sample pipettes and to 2 tubes with the reagent pipettes. These last four tubes control for the presence of contaminating DNA in the reaction mix and the pipettes . Once all sample DNA, control DNA and control water had been added, the reaction mix was overlaid with 100µl light mineral oil (Sigma). The reaction tubes were than closed and moved from the set-up area to a separate room, held under negative pressure, where the thermal cycling was carried out using a Perkin Elmer 480 thermocycler (Applied Biosystems).

2. b. 2. PCR cycling conditions

After a single 4 minute denature step at 94°C, 35 cycles of denaturation (usually 94°C for 20 seconds), primer annealing (between 50 - 60°C for 20 seconds, depending on the parameters of primer construction for each viral or human DNA PCR, see table 2.1) and extension of primer sequence (usually 72°C for 20 seconds) were performed. At the completion of cycling the reactions were maintained at 72°C for 7 minutes to ensure all primer extension was complete. First round products were held at 1°C on the thermocycler until required.

The second round (nested) PCR reaction mixture was identical to the first except that the volume of reaction mixture present in each assay tube was 24 µl. Again these tubes were transported to the thermocycler after set-up in a different room. Using filter tips 1 µl of the first round reaction was transferred to act as a template for the second round reaction. In the second round reaction only 25 cycles of amplification were required, the cycling times were the same as for the first round (anneal temperatures for all the second round primers are given in table 2.1).

2. b. 3. Visualisation of nested PCR products

After the two rounds of PCR the amplified DNA was analysed by ethidium bromide gel electrophoresis (Sambrook *et al.,* 1989). A 100 ml 2% agarose (Promega) gel was prepared, to which 0.5 mg / ml ethidium bromide (Sigma) was added when the gel had cooled to below 60°C. Sample combs were inserted and the gel allowed to set for 30 minutes at room temperature. The sample combs were removed from the gel to provide wells for addition of the PCR product, 10 µl of each PCR product was added directly for electrophoretic separation. To at least one well in each row a molecular weight marker was added so that the size of any PCR product could be estimated.

The marker used for all PCR reactions was Φx174 digested with *Hae* III (Gibco-BRL), 0.3 µg of marker was added per well, after first mixing with 2 µl gel loading buffer (Appendix A) and 7 µl water. The agarose gel, complete with loaded PCR products and marker, was run in an electrophoresis tank (Flowgen) filled with 1x TAE (from 50x stock, Appendix A) at between 60 and 70 mA for 1 hour. After electrophoresis the gel was removed and placed on a UV transilluminator (Flowgen) for visualisation at 302 nm.

2. b. 4. Nested PCR for the detection of herpesvirus DNA

The principles of the nested PCR are described in section 1. b. 3. iii). Nested primers for detection of CMV, HSV-1, HSV-2, VZV, EBV, HHV6 and HHV7 DNA were available for use, their sequences and the cycle conditions used for each are described in table 2.1. Details of the development of the nested PCR's for detection of HHV8 DNA and human DNA are described in section 3. a. 2. and 3. a. 3. The final protocols for each are described in this section, the methods used for oligonucleotide primer design are described below in section 2. b. 5.

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All primers used were synthesised by OSWEL and supplied at a concentration of approximately 0.3 mM in distilled water. Upon receipt primer solutions were adjusted to 0.5 mg / ml and stored in aliquots at -20°C. With such storage the primers have remained stable for over four years.

2. b. 5. Design of nested PCR primers for amplification of human DNA and HHV8 DNA

A single pair of oligonucleotide primers previously used for detection of the human gene pyruvate dehydrogenase were available (Koike, 1990). This sequence is present as a single copy gene in the human genome allowing the

Table 2. 1. Primer sequences, anneal temperatures and expected product sizes for herpesvirus nested PCR and human nested PCR.

Virus (gene)	Nucleotide location	Primer sequence (5'-3')	PCR round	Anneal temp (°C)	Product size (bp)
HSV-1 (gD)	19-43 239-218	ATC ACG GTA GCC CGG CCG TGT GAC A CAT ACC GGA ACG CAC CAC ACA A	1	60	221
	51-71 188-166	CCA TAC CGA CCA CAC CGA CGA GGT AGT TGG TCG TTC GCG CTG AA	2	50	138
HSV-2 (gG)	363-386 546-524	TCA GCC CAT CCT CCT TCG GCA GTA GAT CTG GTA CTC GAA TGT CTC CG	1	60	183
	422-441 522-503	AGA CGT GCG GGT CGT ACA CG CGC GCG GTC CCA GAT CGG CA	2	50	100
VZV (gene 29)	51067-51087 51338-51315	ACG GGT CTT GCC GGA GCT GGT AAT GCC GTG ACC ACC AAG TAT AAT	1	60	272
	51091-51111 51298-51279	ACC TTA AAA CTC ACT ACC AGT CTA ATC CAA GGC GGG TGC AT	2	50	208
CMV (gB)	1942-1966 2091-2067	GAG GAC AAC GAA ATC CTG TTG GGC A GTC GAC GGT GGA GAT ACT GCT GAG G	1	58	150
	1967-1989 2066-2044	ACC ACC GCA CTG AGG AAT GTC AG TCA ATC ATG CGT TTG AAG AGG TA	2	50	100
EBV (large internal repeat)	1347-1366 1517-1498	GAG ACC GAA GTG AAG GCC CT GGT GCC TTC TTA GGA GCT GT	1	50	171
	1399-1421 1495-1476	GCC AGA GGT AAG TGG ACT TTA AT GAG GGG ACC CTG AGA CGG GT	2	54	97
HHV-6 (13R)	17627-17603 17405-17429	AAG CTT GCA CAA TGC CAA AAA ACA G CTC GAG TAT GCC GAG CCC CTA ATC	1	60	223
	17602-17580 17430-17452	TGT TAG GAT ATA CCG ATG TGG CGT TGT TAG GAT ATA CCG ATG TGG CGT	2	60	173
HHV8 (minor capsid protein)	1-20 233-215 (17)	AGC CGA AAG GAT TCC ACC AT TCC GTG TTG TCT ACG TCC A	1	58	233
	22-41 193-174 (17)	GTG CTC GAA TCC AAC GGA TT ATG ACA CAT TGG TGG TAT AT	2	50	171
Human (PDG)	4296 4316 4482-4462	GGG TAT GGA TGA GGA GCT GGA TCT TCC ACA GCC CTC GAC TAA	1	56	186
	4339-4360 4461-4438	CTT GGA GAA GAA GTT GCC CAG T CCT ACA ATT AAG AGT TGA TCC CT	2	56	122

use of the PCR for the accurate quantification of human genome load in a clinical sample (experimentation detailed in section 3. a. 3. iii.). A pair of nested primers was designed by analysis of the nucleotide sequence between the two existing primers using the PCGene database, thus ensuring the specificity of the nucleotide sequences to the human genome.

A single pair of primers for detection of HHV8 DNA were already published (Chang *et al.*, 1994). These two oligonucleotides were designed to amplify the DNA sequence of a 233 bp section of the HHV8 genome termed KS330(233). The entire sequence of KS330(233) was known allowing design of two nested primers sited internal to the existing set. The primers were analysed using the EMBL FASTA Server nucleotide sequence analysis program and found to be specific to the KS330(233) sequence.

2. c. Preparation of DNA from clinical material for analysis by nested PCR.

A large variety of clinical samples were analysed throughout this thesis, for most types of sample existing preparation protocols were available, however details of optimisation of the analysis of herpesvirus DNA in ocular fluid and the analysis of HHV8 DNA in bronchoalveolar lavage (BAL) fluid are contained in sections 3. e. 3. and 3. a. 5. respectively. The final protocols for each are described in this section.

2. c. 1. Extraction of DNA from venous blood

Two methods were used to obtain DNA from peripheral venous blood. For each method the starting material was either 5 ml venous blood collected in an EDTA-containing vacutainer blood tube (Beckton-Dickinson) or 10 ml venous blood mixed with preservative-free heparin (12-30 i.u. / ml). Upon receipt the tube was centrifuged (400g for 10 minutes) to separate all cellular material

from the plasma (upper) fraction. Plasma was removed for storage in 1 ml aliquots at -20°C in 1.5 ml screw-topped tubes (Sarsted). The volume of plasma removed was replaced with PBS (Appendix A) and a plastic Pasteur pipette (BDH) was used to ensure thorough resuspension of the blood cells.

The first method for DNA extraction from peripheral blood was based upon that adapted by Kaye *et al.* (1991). After resuspension in PBS the blood was mixed in equal volume with a glycerol-based freezing medium (glycigel, Appendix A) for storage in 1 ml aliquots at -20°C. This freezing medium allows the blood sample to undergo a cycle of freezing and thawing without lysis of the red and white blood cells. Upon removal from -20°C the sample was thawed in a 37°C water bath and the cells pelleted by centrifugation (8,000g for 5 minutes). The supernatant was removed and 1 ml lysis buffer (LB, Appendix A) added after which the tube was vortexed to mix the LB with the blood cells. The LB was formulated to allow lysis of the erythrocyte and leucocyte cell membranes while the leucocyte nuclei, containing the herpesviral DNA, remained intact. Three more cycles of pelleting, addition of lysis buffer and vortex were then used to clear the sample of all contaminating erythrocytes leaving the leucocyte nuclei visible as a small white pellet. The leucocyte nuclei were then lysed by addition of 100µl extraction buffer (EB, Appendix A) and incubation for 2 hours in a 60°C water bath. Finally the samples were boiled for 10 minutes to inactivate the protinase K activity of the EB and between 1 and 10µl added to the nested PCR.

The second method by which DNA was extracted from venous blood was by use of a commercial DNA preparation kit (Qiagen blood kit, Qiagen). With this system EDTA-treated whole blood may be stored, without prior preparation, at -20°C. When required the blood was thawed, mixed to ensure homogenicity and 50µl mixed with 150µl PBS before addition of a lysis / extraction buffer. After incubation for 15 minutes in a 70°C water bath the disrupted material was added to a silica column designed to capture DNA molecules. The column was washed twice to remove contaminants and the

DNA eluted in 50µl water. The DNA solution was then boiled for 10 minutes, cooled on ice and between 1 and 10 µl added to the nested PCR.

2. c. 2. *Extraction of DNA from plasma and serum*

Plasma was separated from whole venous blood as described in section 2. c. 1. and stored at -20°C. Serum was prepared by centrifugation of whole clotted blood (400 g for 10 minutes) and was also stored at -20°C. DNA was prepared from both plasma and serum by addition of 0.5 ml water to 0.5 ml sample, mixing by vortexing and boiling for 10 minutes. The mixture was cooled on ice and pelleted (usually 14,000 g for 30 minutes) until a clear supernatant was visible as a separate top phase above the coagulated protein. Between 1µl and 10µl of the clarified supernatant were added to the nested PCR.

2. c. 3. *Extraction of DNA from cultured cells*

Cells of the BCP-1 or HBL-6 lines were pelleted by centrifugation (400g for 10 minutes) and washed once in PBS before resuspension in approximately one tenth original volume glycigel EB. The cells were incubated at 60°C for 30 minutes to allow disintegration of the cell membranes and DNA was released from the cell nuclei by boiling for 10 minutes. After cooling on ice the extract was clarified by centrifugation (8,000g for 10 minutes) and between 1 and 10µl of the supernatant added to the nested PCR.

2. c. 4. *Extraction of DNA from solid tissue samples*

Tissue samples, including skin, post-mortem biopsies of brain and solid hypervascular sarcoma samples of varying type were snap frozen dry at either

-70°C or in liquid nitrogen for storage. After thawing, approximately 0.5cm^3 of tissue was ground under liquid nitrogen with a pestle and mortar (BDH). 1ml of an SDS-based extraction buffer (SDS-EB, Appendix A) containing proteinase K was used to resuspend the ground material which was then transferred to a 1.5 ml screw-top tube. After incubation over-night, at 60°C, purified DNA was prepared by addition of 0.5 ml of the extract in SDS-EB to a mixture of buffer-saturated phenol (Appendix A) / chloroform (BDH, 99.5%), and isoamyl alcohol (BDH), in a 24 : 24 : 1 ratio. Thorough mixing was ensured using a horisontal shaker for 10 minutes and the extracted sample was then centrifuged (14,000 g for 10 minutes). The clear, upper, aqueous phase resulting after centrifugation was removed to a fresh 1.5 ml tube and the extraction process repeated with addition of a further 0.5 ml buffer saturated phenol / chloroform / isoamyl alcohol. After this second extraction the recovered aqueous phase was added to 0.5 ml chloroform / isoamyl alcohol in a 24 : 1 ratio in a fresh tube and the extraction process repeated once more. After the final extraction DNA in the aqueous phase was purified by ethanol precipitation in the presence of excess salt (Sambrook *et al.*, 1989). The aqueous phase was added to 1 ml ethanol which had been cooled to -20°C (ice-cold) and contained 120 mM NaCl. The tube was mixed by vortexing and stored at -20°C overnight to allow DNA precipitation. Precipitated DNA was pelleted from the solution at 4°C (14,000g for 30 minutes). The DNA pellet was washed twice in ice-cold 100% ethanol and once in ice-cold 70% ethanol, between each wash the DNA was repelleted (14,000g for 10 minutes). After the final wash the majority of ethanol was removed using a fine-tipped Pasteur pipette (BDH). The tube was left for 20 minutes in a 37°C incubator to evaporate any remaining ethanol. The DNA pellet was resuspended 100µl TE buffer (Appendix A) and the concentration of DNA recovered estimated using the equation: 1 OD_{260} = 50g / ml double-stranded DNA (1cm light path).

2. c. 5. Extraction of DNA from saliva, throat swab transport medium, urine and cerebro-spinal fluid.

Saliva samples were collected directly into a sterile container after first rinsing the mouth with 10ml sterile saline solution. Cells in the saliva sample were pelleted by centrifugation (8,000 g for 10 minutes) and the resulting cell-pellet and supernatant stored separately. DNA from the supernatant was prepared by boiling for 10 minutes and cooling on ice. The boiled supernatant was then added directly to the nested PCR (Saiki *et al.*, 1986). The same storage preparation and DNA extraction method was used for throat swab transport medium (Appendix A), urine and cerebro-spinal fluid (CSF) collected by lumbar puncture. All samples were transferred to the laboratory in sterile containers, usually on the same day they were taken. If same-day transport was not possible they were stored at 4°C until transport could be arranged.

2. c. 6. Extraction of DNA from bronchoalveolar lavage fluid (BAL)

Bronchoalveolar lavage fluid (BAL) was taken during fibreoptic bronchoscopy by addition of 300 ml sterile saline to the bronchial tree and recovery of approximately 100ml by aspiration. The BAL fluid was transported to the laboratory within 4 hours in a sterile container. BAL fluid was stored at -20°C both after separation into cell-pellet and supernatant fractions by centrifugation (8000g for 10 minutes) and unfractionated. For analysis by nested PCR 1 ml whole BAL fluid was boiled for 10 minutes, cooled on ice, centrifuged (8,000g for 10 minutes) and 1-50µl of the supernatant added directly to the nested PCR. DNA was extracted from cell-free virus particles in BAL supernatant by ultracentrifugation (100,000g for 30 minutes) of the BAL supernatant and extraction of the resultant virus pellet by the phenol / chloroform process (2. c. 4.)

2. c. 7. Extraction of DNA from ocular fluids (vitreous and aqueous)

Ocular fluids; aqueous fluid from the anterior chamber of the eye and vitreous fluid from the posterior chamber of the eye were usually received already recovered from the eye. However in the study of herpesviral DNA in post-mortem ocular fluids (section 3. e.) whole cadaver eye-balls were received from which the two ocular fluids required recovery using a fine-bore needle (Teruma, 19 gauge) and 5 ml syringe (Sabre). Following receipt or recovery the ocular fluid was centrifuged (8,000 g for 10 minutes) to separate cells from supernatant and the two fractions stored separately at -20°C. DNA was prepared from the ocular fluid supernatant by the method of boiling described for saliva, CSF, urine and throat swab medium supernatant and 1 to 10 µl of the supernatant added directly to the nested PCR.

2. c. 8. Extraction of DNA from faeces.

Faecal material was suspended in water to give a 10% slurry and mixed by vortexing. The slurry was centrifuged (1,500g for 10 minutes) to pellet gross contaminants and 0.5 ml of the supernatant re-pelleted by ultracentrifugation (100,000g for 10 minutes). The ultracentrifuged pellet was resuspended in 20µl water. This was disintegrated by boiling for 10 minutes, cooled on ice, and 1 to 10 µl of the released DNA added directly to the nested PCR.

2. d. Indirect immunofluorescence (IIF) assay for detection of anti-HHV8 IgG

The indirect immunofluorescence (IIF) assay used for the detection of anti-HHV8 IgG antibodies was based upon an IIF assay developed by Simpson and colleagues and used the same HHV8-infected cell line BCP-1 (Simpson *et al.*, 1996). This technique is similar to a number of previously described IIF assays for detection of IgG antibody response to other human herpesviruses such as EBV and HHV6 (Gleaves *et al.*, 1984 and Fox *et al.*, 1988).

2. d. 1. Preparation of BCP-1 IIF slides and sera

BCP-1 cells were grown on feeder cell layers of HEL cells as described in section 2. a. 2. To prepare the IIF slides 2-3 ml of a BCP-1 culture at approximately 5 x 10^6 cells / ml was removed from culture and pelleted by centrifugation (400g, 10 minutes) in a 50 ml screw-top centrifuge tube (Falcon). The cell pellet was washed in 30 ml PBS. After washing the cells were again pelleted and then resuspended in a fixing solution of 4% paraformaldehyde for 10 minutes at room temperature. After a further wash in 30 ml PBS the cells were incubated for 10 minutes at room temperature in 1% Triton X-100 (Sigma) / PBS. This detergent treatment permeablises the BCP-1 cell membranes to allow intracellular entry of anti-HHV8 serum antibodies for interaction with virus-encoded antigens. After a final PBS wash the fixed, permeablised cells were resuspended in 1ml PBS and 10µl of the resuspended cells added to each well of a 12-well Teflon-coated slide (C.A. Henley). An initial harvest of 1 x 10^7 cells enabled preparation of 10 slides with each slide well containing approximately 5 x 10^4 - 1 x 10^5 cells. This concentration of cells was found to be optimal for visualisation of fluorescent signal in the IIF assay. After the cells had been added to the slide wells they were left for 10 - 15 minutes to dry at room temperature. Slides prepared by this method were best used the same day, long-term storage at -20°C was found to adversely

effect the performance of the IIF assay by reducing the intensity of fluorescence signal.

In the published description of the anti-HHV8 IgG IIF assay (Simpson *et al.*, 1996) sera were tested at an initial dilution of 1 in 150 in PBS. However, it was found possible to reduce this dilution to 1 in 50 by using 3% FBS / PBS as a diluent (personal communication D. Whitby, Institute of Cancer Research, London) without the introduction of non-specific fluorescent signal. This initial serum dilution was therefore used throughout these studies to ensure maximum assay sensitivity. Any samples which did produce non-specific signal were reanalysed at dilutions of 1 in 50, 1 in 100 and 1 in 150.

2. d. 2. Detection of anti-HHV8 IgG by IIF

Ten microlitres of serum diluted 1 in 50 in PBS / 3% FBS were added in duplicate to the fixed BCP-1 cells on multispot slides and distributed with a sterile pipette tip to ensure even covering of the cells. Slides were incubated in a moist chamber for 40 minutes at room temperature before removal of the serum by washing 6 times in PBS / 3% FBS. Fluorescin conjugated anti-human IgG (Dako) was diluted 1 in 200 in PBS / 3% FBS and 25µl then added to each well. After a further 40 minute incubation at room temperature the slide was again washed 6 times in PBS / 3% FBS and excess wash solution removed by drying in air. The slides were mounted in few drops of a mounting solution (Appendix A), a cover slip (BDH) added and the cells examined using a fluorescent microscope (Carl Zeiss). Known positive and negative sera were included in each experimental set and examined first to ensure assay validity.

Positive staining for anti-HHV8 IgG was detected as a punctate, predominantly (although not exclusively) nuclear staining of the HHV8 open reading frame (orf) 73 latent nuclear antigen (LNA) (fig. 2. 3. b) (Rainbow *et al.*, 1997). Sera not containing anti-HHV8 IgG also produced an

Figure 2. 3. a. Negative IIF anti-HHV8 IgG signal with BCP-1 cell line

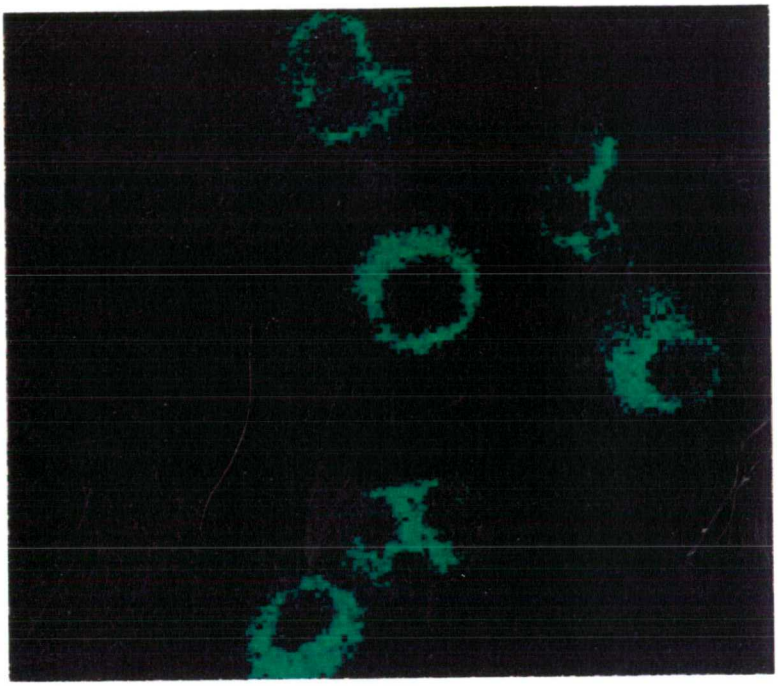
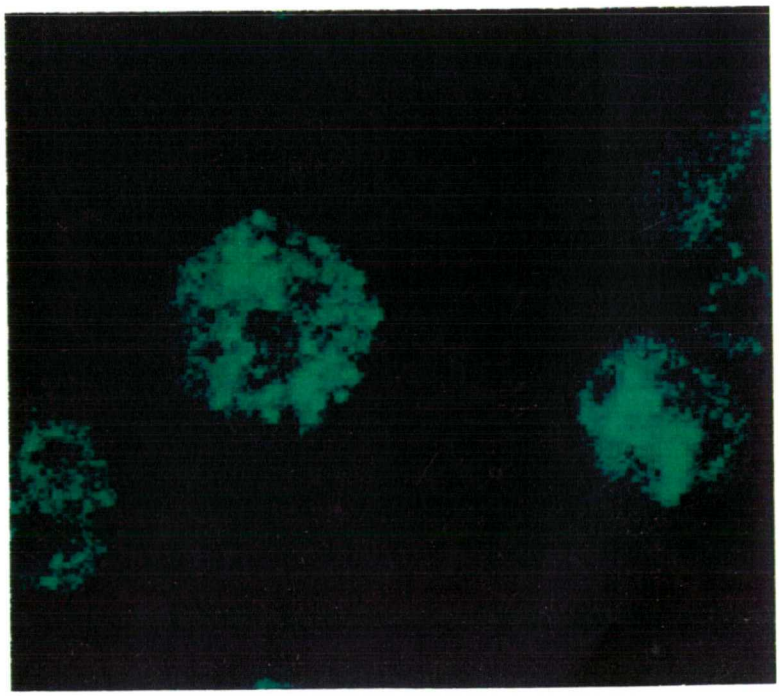


Figure 2. 3. b. Positive IIF anti-HHV8 IgG signal with BCP-1 cell line



immunofluorescence signal, however this was recognised by a profuse, background membrane signal which showed none of the distinct nuclear staining pattern exhibited by positive serum (fig. 2. 3. a). When analysed sera were scored either +, - or +/-. If equivocal the analysis of that sera was repeated at a dilution of 1 in 100, as well as at 1 in 50 and 1 in 150.

2. e. Recombinant enzyme-linked immunoabsorbant assay (ELISA) for the detection of anti-HHV8 IgG.

The HHV8 orf 65 gene product was chosen as a prospective antigen target for use in the detection of anti-HHV8 antibodies because of its nucleotide sequence homology with the EBV gene product BFRF3 (van Grunsven *et al.*, 1994). BFRF3 is a capsid protein which shows reactivity with over 90% of sera from EBV-infected individuals. However as the level of homology, at the amino acid level, of BFRF3 and HHV8 orf 65 was only 26% it was felt that cross-reactivity between anti-EBV antibodies and anti-HHV8 antibodies would be avoided (personal communication Thomas Schulz, University of Liverpool).

2. e. 1. Generation of recombinant HHV8 antigen

Two segments of the orf 65 protein were expressed, fused to the carboxyterminal end of the mouse dihydrofolate reductase gene product, under the control of a T7 promoter in the expression vector pQE 42 (Qiagen). Orf 65.1 (aminoacids 2 - 170 of orf 65) and orf 65.2 (aminoacids 86 - 170) were expressed separately. The expression construct contained a histidine tag at the amino-terminal end of the fusion protein allowing purification by affinity chromatography on nickel resin. The orf 65.2 recombinant protein was chosen for assay development, this polypeptide shares only 18 aminoacids (21% identity) with the equivalent region of BFRF3 and when 10 high-titre anti-

EBV sera were analysed no cross-reactivity was detected (Simpson *et al.*, 1996).

The recombinant orf 65.2 antigen was constructed and produced by colleagues in the Department of Microbiology, University of Liverpool. It was received in 7M urea, on dry ice, to ensure stability. Upon receipt the antigen was aliquoted in 0.5 ml lots and placed at -70°C in 1.5 ml tubes for long-term storage.

2. e. 2. Preparation of sera

Prior to the analysis of sera for presence of anti-HHV8 IgG by the orf 65.2 recombinant ELISA the serum was diluted 1 in 100 in 5% dried skimmed milk (Marvel) / PBS-Tween-20 (Sigma) (PBS-T), (ELISA blocking buffer, Appendix A). 100µl volumes of this diluted sera were analysed in duplicate by the ELISA.

2. e. 3. Detection of anti-HHV8 IgG using the orf 65.2 recombinant ELISA

Ninety-six well ELISA plates (Maxisorb, Nunc) were coated with 100µl purified orf 65.2 recombinant protein after pre-dilution in 0.1M / L $NaHCO_3$, pH 8.5 (usually 1 in 60, depending on batch concentration). The plates were incubated overnight at room temperature in a moist chamber. After incubation of the antigen the plate was washed 6 times in PBS-T and tapped dry on tissue paper. The plate was then blocked with 200µl ELISA blocking buffer (ELISA-BB), and incubated for 2 hours at room temperature. In this state the plate could be stored at 4°C for later use, however it was noted that assay performance was significantly improved if plates prepared the same day were used in each assay.

When required the ELISA-BB was removed and the plate washed once in PBS-T before addition of the test sera pre-diluted in ELISA-BB. The sera was incubated on the plate for 1 hour at room temperature. Test sera was removed by washing 6 times in PBS-T before addition of a second antibody. The second antibody was an alkaline phosphatase-conjugated, affinity-purified, goat anti-human IgG (Dako) diluted 1 in 1000 in ELISA-BB with 1% goat sera (Sigma). 100µl of the conjugated antibody was added to each well, incubated for 1 hour at room temperature, and then the wells washed 6 times in PBS-T. After tapping the plate dry the wells were washed once in 200µl 1M diethanolamine solution before addition of 200µl of a 1 mg/ml solution of dinitrophenyl phosphate (Sigma) in 1M diethanolamine. The plate was incubated at 37°C for approximately 30 minutes before reading of the colorimetric reaction spectrophotometricaly at 405 nm. If after 30 minutes incubation the positive control sera gave an optical density (OD) of below 1.0 the plate was transferred back to the 37°C incubator and the OD checked every 5 minutes until a sufficiently strong colorimetric reaction had been achieved.

To determine the cut-off of positive and negative sera in each assay 3 anti-HHV8 IgG negative sera were present on the ELISA plate and their mean OD at 405 nm determined. A serum sample was deemed to contain anti-HHV8 IgG if the average OD of the two duplicates of that sample was greater than 3 times the mean of these negative control samples.

2. f. Nested PCR herpesvirus multiplex assay

There are a variety of methods available for the detection of herpesvirus infections in the clinical setting (see section 1. b.). Traditionally these have been based upon the relative ease with which certain members of the herpesvirus family can be cultured *in vitro* directly from patient material. However with the development of transient culture systems such as the DEAFF test, viral antigenaemia detection and the use of molecular diagnostic techniques such as PCR, it is now possible to detect and measure extremely low levels of herpesvirus. These later molecular amplification assays have been of particular advantage in identifying herpesvirus involvement in neurological and ocular diseases of the immunocompromised host (see sections 3. a. 6. and 3. e.).

The ultra-sensitive herpesvirus detection assay used in the Department of Virology, UCLMS since 1992 has been the nested PCR (see section 2. b.). Oligonucleotide primers are available for detection of all eight human herpesviruses, although analysis for human herpesvirus 7 (HHV7) DNA is rarely requested. In order to reduce the need for multiple individual PCR analysis on clinical samples a multiplex nested PCR (mPCR) was developed. This assay allows simultaneous amplification of HSV-1, HSV-2, VZV, EBV, CMV and HHV8 DNA with the detection of amplicons by an enzyme-linked oligonucleotide (ELONA) assay based upon an assay originally proposed for the quantification of hepatitis C virus RNA load (Whitby and Garson, 1995).

2. f. 1. Multiplex PCR assay conditions

A wide range of PCR conditions were investigated in order to maximise the sensitivity of the mPCR-ELONA assay. Optimisation of the assay was determined jointly by myself and by Mr. Stuart Kirk, Department of Virology, UCLMS.

These optimisation experiments are not included in this thesis but can be found described in Kirk *et al.* (submitted *J Clin Micro,* 1998).

In the first round amplification reaction oligonucleotide primer pairs for amplification of HSV-1, HSV-2, EBV, VZV, CMV and HHV8 DNA were present in the same concentration as for the individual PCR assays (see section 2. b. 3.). The formulation and volume of the PCR reaction buffer and concentration of nucleotides used was also unchanged from the individual PCR assays. The amplification enzyme *Taq*gold (Applied Biosystems, 1.25 units per tube) was used instead of *Taq* to improve the specificity of derived amplification products. To allow activation of the *Taq*gold an initial denaturation step of 95°C for 12 minutes was employed before both rounds of the mPCR. The optimum cycle conditions for the first round were; 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds for 40 cycles, followed by a final 7 minute, 72°C extension cycle.

The optimal cycle conditions for the second round, following the 95°C activation step were; 95°C for 30 seconds, 46°C for 30 seconds and 72°C for 30 seconds for 32 cycles, followed by a 7 minute extension cycle at 72°C. As for the first round reaction a 50 µl reaction volume was used and the reaction buffer and nucleotide concentration remained the same as for the individual PCR reactions. Again 1.25 units of *Taq*gold were present in each reaction tube. The oligonucleotide primers used in the mPCR second round were the same as in the second round of each of the individual PCR assays, however the primers were present at the lower concentration of 30 ng each and one of every pair had a biotin molecule covalently-attached to its 5' terminus. lµl of the mPCR first round amplification product was used to prime the second round reaction.

2. f. 2. Multiplex PCR amplicon detection- the enzyme-linked oligonucleotide assay (ELONA)

An enzyme-linked oligonucleotide assay (ELONA) based on that developed by Whitby and Garson, was modified to produce a colorimetric signal (figure 2.4.) (Whitby and Garson *et al.,* 1995). Clear 96-well microtitre plates (Maxisorb, Nunc) were coated with 5µg / ml streptavidin (Sigma) in a carbonate / bicarbonate coating buffer (pH 9.6, Appendix A) at room temperature overnight. After aspiration of the coating buffer the wells were blocked with 0.2% BSA / PBS at 4°C overnight, or room temperature for 1 hour. Plates blocked in this fashion were stable for up to two months if covered with a plastic plate-sealer (ICN-Flow) and stored at 4°C.

For the detection of the mPCR amplified products the blocking buffer was aspirated and 5 µl of the mPCR second round product added to the plate, diluted 1:20 in an ELONA sample diluent (ELONA-SD, Appendix A). The product was incubated on the plate for 30 minutes at 45°C. In the initial "screening-ELONA" each nested mPCR product was added to a single microtitre well. After product incubation the plate was washed 10 times in Tris/Tween/Azide (TTA, Appendix A) wash buffer before final aspiration and the addition of 100µl 0.15M NaOH for 2 minutes at room temperature. This incubation was followed by a further 10 washes in TTA wash buffer.

Anti-sense oligonucleotide probes were designed of complimentary internal sequences to each of the 6 target virus mPCR products (table 2. 2.). These probes were modified to have the enzyme horse radish peroxidase (HRP) covalently attached at their 5' end. The working dilution of each probe was determined empirically, however a dilution of 1 in 20,000 in probe diluent (ELONA-PD,

Figure 2. 4. Diagrammatic representation of the optimised protocol for the detection of mPCR amplification products by ELONA

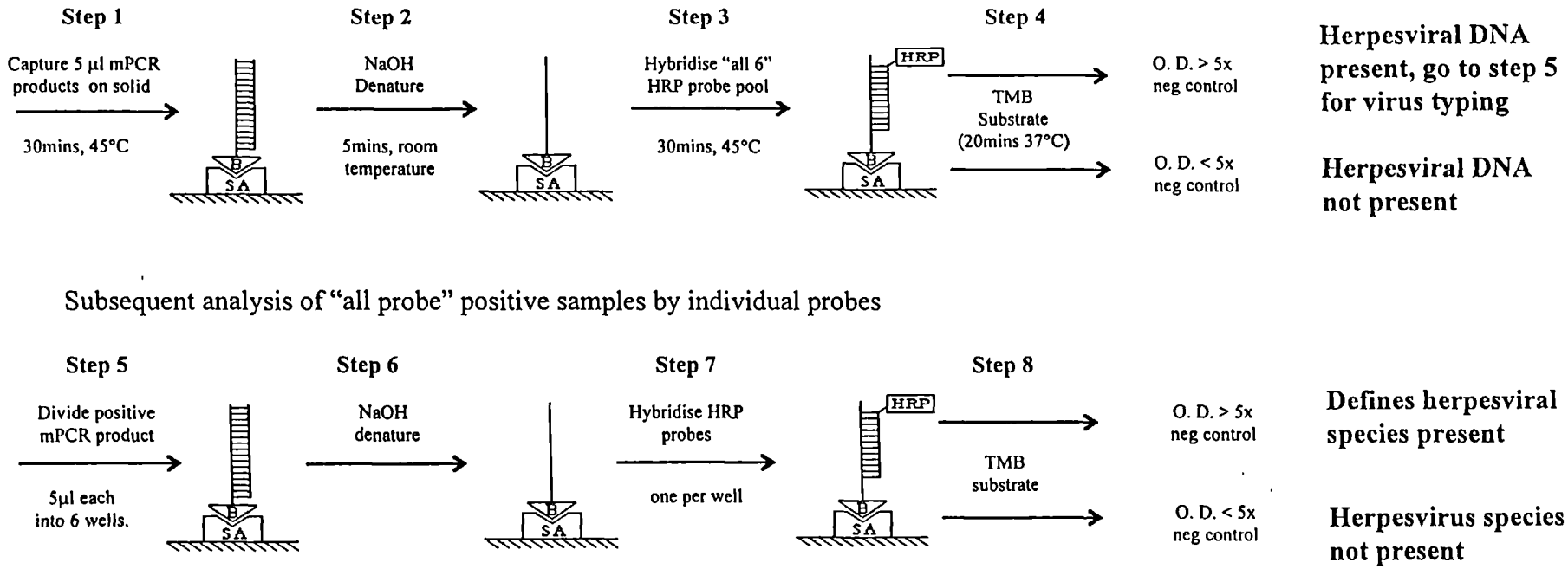


Table 2. 2. Nucleotide sequences and location of 5'-horse radish peroxidase-labelled oligonucleotide probes used for the ELONA detection of herpesvirus multiplex PCR products.

Virus (gene)	Probe location on genome	Probe sequence (5'-3')
HSV-1 (gD)	107-89	CTC CTC CTC GTA AAA TGG C
HSV-2 (gG)	493-474	GCA TTT ACG AGA GCG TAC
VZV (gene 29)	51154-51135	CGT GTT TGC CTC CGT GAA AG
CMV (gB)	2013-1996	GAT GAA GAT CTT GAG GCT
EBV (large internal repeat)	1454-1437	GGG TGT GGT GGA GTG TTG
HHV8 (minor capsid protein)	109-92	ACA CCA ACA GCT GCT GC

Appendix A) was usually optimal. In the "screening- ELONA" 100µl of a pool of all 6 probes, diluted in ELONA-PD, was added to each well of the plate containing mPCR products and incubated at 45°C for 30 minutes. Unbound probe was removed by washing 10 times, with 1 minute soaks between each wash, in 0.05% Tween-20./ PBS, 100µl of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) in tri-sodium citrate and hydrogen peroxide diluent (Murex Biotech) was then added and the plate incubated at 37°C for 20 minutes. The colorimetric reaction was stopped using 2N H_2SO_4.

The optical densities for each well were measured spectrophotometricalty at 450nm and for each sample a test : negative ratio was calculated by dividing the optical density of the test well by the average of the optical densities of the wells containing the water negative controls. No substrate blank was included in this calculation and any well having a test : negative ratio of 5 or greater was defined as containing a detectable herpesvirus mPCR product. By using a pool of all 6 herpesvirus-amplicon specific probes the "screening-ELONA" assay was thus able to identify mPCR products containing any amplified herpesviral DNA. However, the screening-ELONA was unable to determine the particular species of herpesviral DNA present. For species typing of the screening-ELONA-positive mPCR products 5µl, in 95 µl ELONA-SD, of the remaining mPCR nested product from that reaction were added to each of 6 separate streptavidin-coated wells and incubated as before. The working concentrations of the 6 probes were then made up individually and, after NaOH treatment of the bound amplicons, one probe added to each of the 6 wells. ELONA detection was carried out as before, with any well, or wells, showing a test : negative optical density ratio greater than 5 indicating the virus type, or types, present within the original sample.

Section 3: Results

3. a. Association of HHV8 with HIV-associated KS

3. a. 1. Introduction

This first results section deals with the development of nested PCR reactions for the detection of HHV8 and human DNA, and the use of both these assays to investigate the prevalence of HHV8 DNA in a variety of patient groups:

1. In the peripheral blood of HIV-infected individuals with, and without, KS and of healthy blood donors.

2. In the bronchoalveolar lavage (BAL) fluid of HIV-infected patients with and without pulmonary KS.

3. In the CSF of HIV-infected patients with and without central nervous system (CNS) lymphoma.

4. In the semen of HIV-infected gay men and a large cohort of healthy UK semen donors.

These studies were performed to assess the distribution of HHV8 genome in a range of individuals at varying risk of HHV8 infection, and to investigate earlier reports of an association between HHV8 and KS.

3. a. 2. Nested PCR for the detection of HHV8 DNA

As described in section 1. b. 3. the nested polymerase chain reaction (PCR) is a technique for the amplification of specific sequences of DNA (Simmonds *et al.*, 1990). The repeated cycles of DNA amplification occurring in the nested PCR reaction allow easy detection of as little as a single starting molecule of target DNA making this technique ideal for analysis of herpesviral DNA in clinical samples (Fox *et al.*, 1991 and Wakefield *et al.*, 1992). A detailed methodology for all nested PCR reactions used in this thesis can be found in section 2. b. and the theory behind the design of oligonucleotide primers for use in nested PCR is described in section 1. b. 3.

3. a. 2. i) selection of primer sequences for the second round of the HHV8 PCR

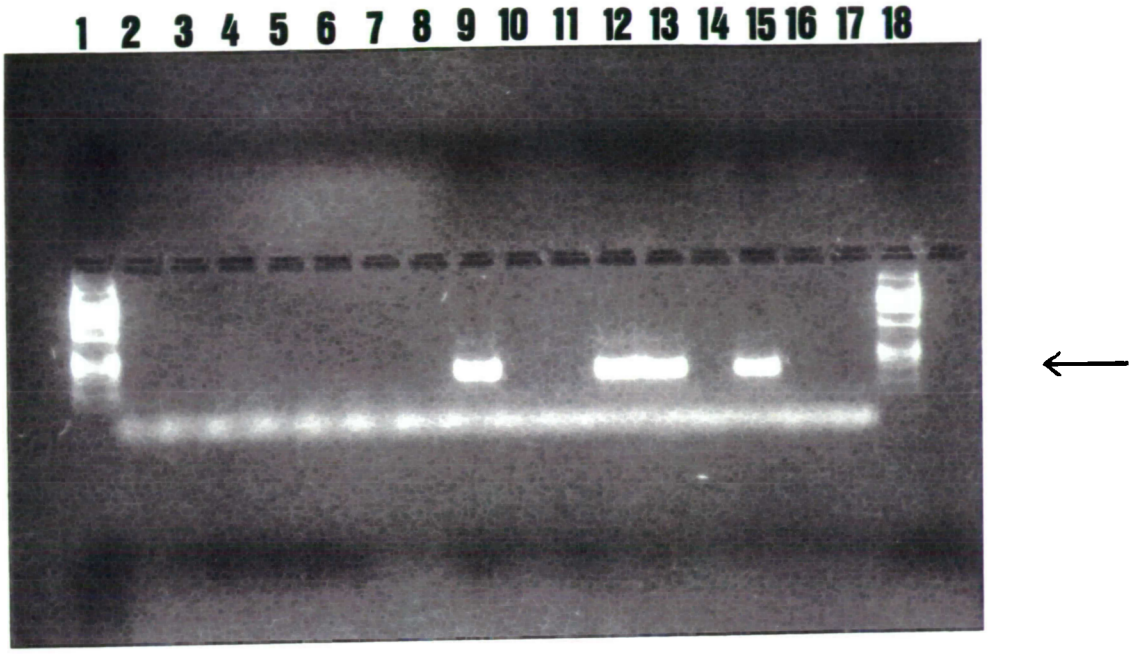
Within the KS330Bam fragment of the HHV8 genome, which contains the KS330(233) sequence of the HHV8 PCR first round amplicon, no significant nucleotide sequence strain variation has been noted. Studies have shown that this KS330Bam fragment is situated in an HHV8 open reading frame (*ORF26*) which has a greater than 50% homology with other known herpesvirus minor capsid genes, especially the minor capsid genes *ORF 26* of HVS and *BDLF 1* of EBV respectively (Russo *et al.*, 1996).The genetic stability of the region enabled second round PCR primers to be designed internal to the first round primers without risk of mismatches affecting the subsequent amplification efficiency of the assay. The two internal primers developed; HHV8 (3) and HHV8 (4) (see table 2.1) were analysed using the EMBL FASTA genome database and found to be specific for amplification of a sequence within the first round amplicon.

3. a. 2. ii). HHV8 nested PCR conditions, PCR specificity and confirmation of correct sequence amplification

The projected size of the amplicons produced by the first and second round PCR amplification for HHV8 DNA, as well as the Tm of the oligonucleotide primers designed, was similar to the PCR reaction for amplification of CMV DNA already in use (table 2.1.). For this reason identical reaction and cycling conditions as for the CMV PCR were selected. When a variety of DNA samples, prepared from glycigel-stored blood taken from patients with cutaneous KS, were analysed by the nested HHV8 PCR all those found to contain HHV8 DNA gave bands of equal high intensity after ethidium bromide gel electrophoresis (fig. 3.1.1). This suggested that the 35 cycles of amplification employed in the first round and the 25 cycles of amplification in the second round were sufficient for use in the HHV8 PCR. The approximate size of both first (230 bp, see figure 3.1.2.) and second (180bp, see figure 3.1.1.) round amplicons was determined by comparison with the Φx174 markers and found to be as expected from the sequence data.

At the time of development of the HHV8 PCR no known positive control DNA was available to include in each PCR reaction series. Therefore a sample from the first reaction series (figure 3.1.1, number 9) which gave an amplicon after both the first and second rounds of amplification was selected. Visualisation of an amplification product after one round of PCR cycling suggested that sample 9 contained a high level of HHV8 DNA. To determine the HHV8 DNA titre of sample 9 a ten-fold dilution series of DNA from that sample was prepared. The last dilution at which HHV8 DNA could still be amplified, when 1μl of each of these dilutions was analysed, was 1 in 10^{-4}, therefore the titre of detectable HHV8 DNA in this sample was approximately 10,000 copies per microlitre. In each subsequent PCR reaction series tubes containing 10, 1 and <1 detectable copies of sample 9 HHV8 DNA were included so that inter-assay sensitivity could be monitored.

Figure 3. 1. 1. HHV8 nested PCR reaction products after 35 cycles of amplification first round and 25 cycles of amplification second round.



Lanes 1 and 18: molecular weight marker - ΦX 174 cut with *Hae III*
band sizes in nucleotides (from bottom up) 72, 118, 194, 234, 271, 310, 603

Lanes 2 - 8, 10 - 11 and 14: samples containing no detectable HHV8 DNA

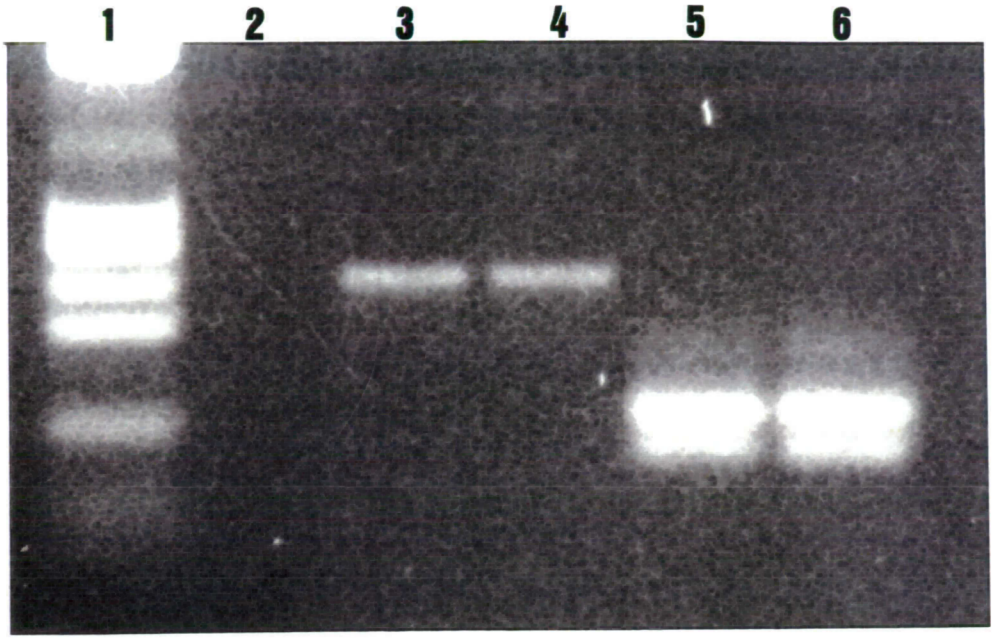
Lanes 9, 12 - 13 and 15: samples containing detectable HHV8 DNA

(lane 9: sample selected for use as HHV8 DNA positive control)

Lanes 16 -17: negative water controls

Product size (180 bp) indicated by arrow

Figure 3. 1. 2. Restriction enzyme digest of first round HHV8 PCR products



Lane 1: molecular weight marker - ΦX 174 cut with *Hae III*
band sizes in nucleotides (from bottom up) 72, 118, 194, 234, 271, 310, 603

Lane 2: empty lane

Lanes 3 and 4: undigested first round product

Lanes 5 and 6: *Puv II* digested first round product

Product size (233 bp) indicated by arrow

To confirm that the amplicon produced by the first round of HHV8 PCR was of the expected sequence a restriction enzyme (RE) digest was performed. Sequence data from Chang and colleagues showed that within the predicted first round amplicon sequence there existed a *Pvu II* RE site, cutting at base 99 of the 233 bp amplicon (Chang *et al.*, 1994). Therefore digestion with *Pvu II* should produce fragments of 99 bp and 132 bp in size when analysed by gel electrophoresis. To determine whether *Puv II* could cut the first round amplicon at this site 60 µl of amplicon was added to 10 units of *Pvu II* (Promega). After a 60 minute incubation at 37°C the digested and undigested products were analysed. As figure 3.1.2. shows the first round amplicon was successfully digested by *Pvu II* to give 2 digestion products which were both found to migrate close to the 118 bp DNA band of the Φx174 markers (figure 3.1.2.). The digestion products were therefore of the predicted size and this showed that the HHV8 first round amplicon contained the *Pvu II* RE site. This proved that the HHV8 PCR amplified a sequence within the KS330Bam sequence of the HHV8 genome.

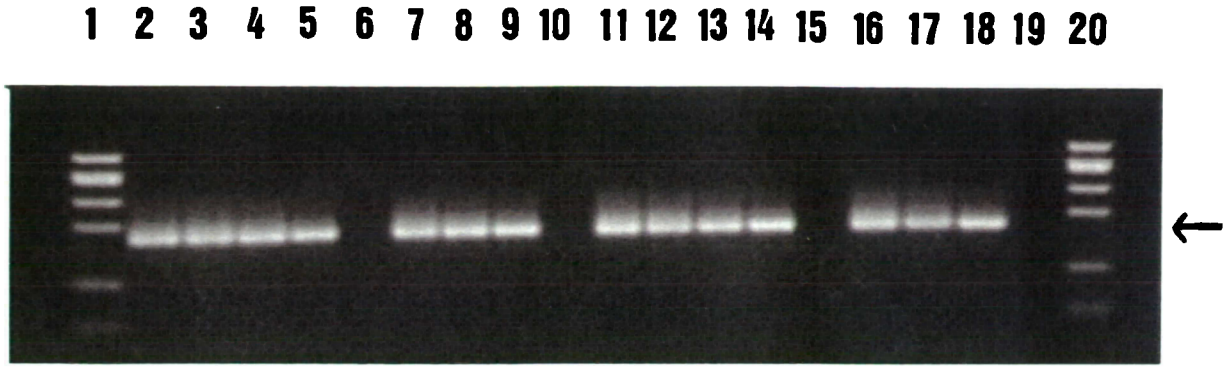
Once correct amplification of HHV8 DNA had been confirmed, samples known to contain human, HSV-1, HSV-2, VZV, EBV, CMV, HHV6 and HHV7 DNA were analysed to ensure that no cross-reactivity would occur with the HHV8 primer sets. Use of primary clinical samples was avoided in case the patient from which they were prepared was co-incidentally HHV8 DNA positive. In all cases tissue culture supernatant was chosen and after completing the HHV8 PCR assay no amplification products were identified from any of these supernatants.

3. a. 2. iii). determination of HHV8 PCR sensitivity

The absolute sensitivity of the HHV8 PCR was determined using DNA extracted from the HHV8 / EBV co-infected cell-line HBL-6. A culture was prepared and found to have a concentration of 1.35 x 10^6 cells / ml. 2ml of these cells were pelleted by centrifugation (800g, 10 minutes), washed in RPMI and resuspended to give a stock of 1 x 10^6 cells / ml. A portion of this stock solution was taken and further diluted 1 : 10 in RPMI. The DNA from 0.5 ml of both the diluted and undiluted stock was prepared by phenol / chloroform extraction. The purified DNA was resuspended in TE (Appendix A) to give two DNA preparations, one with the concentration of 100 cells-worth of DNA per microlitre and the other 10 cells-worth DNA per microlitre.

Ten-fold serial dilution of the prepared DNA was used to find the last dilution which still contained detectable HHV8 DNA. For both DNA preparations it was found that after analysing the stocks in 4 duplicate reaction series the equivalent of 0.1 HBL-6 cells-worth HHV8 DNA could be reproducibly detected by the HHV8 PCR (fig 3.1.3.). The HBL-6 cell-line contains 20-50 copies of HHV8 DNA per cell (personal communication P. Moore, Columbia University) therefore the theoretical absolute sensitivity limit of HHV8 PCR detection was approximately 2-5 HHV8 genomes. The number of cell genomes present in the two DNA solutions was subsequently confirmed to be 100 and 10 respectively by analysis of the human genome load using a human PCR (section 3. a. 3.) to the single copy gene pyruvate dehydrogenase. This level of HHV8 PCR detection sensitivity compares well with that described by Boshoff and colleagues (Boshoff *et al.*, 1995) who also designed a nested PCR for the detection of HHV8 DNA using the KS330Bam sequence. This group found that using similar reaction conditions as described above they were also able to detect 0.1 HBL-6 cell's worth of HHV8 DNA and defined their absolute HHV8 PCR detection sensitivity as 10 copies of HHV8 DNA.

Figure 3. 1. 3. HHV8 DNA detection in ten-fold serial dilutions of extracted HBL-6 cell DNA



Lanes 1 and 20: molecular weight marker - ΦX 174 cut with *Hae III*
band sizes in nucleotides (from bottom up) 72, 118, 194, 234, 271, 310, 603

Experiment I

Lanes 2 - 6: ten-fold dilutions of DNA extracted from 100 HBL-6 cells:

lane 2: 100 cells-worth HBL-6 DNA (c.2000 HHV8 genomes)

lane 3: 10 cells-worth HBL-6 DNA (c.200 HHV8 genomes)

lane 4: 1 cells-worth HBL-6 DNA (c. 20 HHV8 genomes)

lane 5: 0.1 cells-worth HBL-6 DNA (c. 2 HHV8 genomes)

lane 6: < 0.1 cells-worth HBL-6 DNA (< 2 HHV8 genomes)

Experiment II

Lanes 7 - 10: ten-fold dilutions of DNA extracted from 10 HBL-6 cells

lane 7: 10 cells-worth HBL-6 DNA (c.200 HHV8 genomes)

lane 8: 1 cells-worth HBL-6 DNA (c.20 HHV8 genomes)

Lane 9: 0.1 cells-worth HBL-6 DNA (c. 2 HHV8 genomes)

Lane 10: < 0.1 cells-worth HBL-6 DNA (< 2 HHV8 genomes)

Lanes 11 - 19: Experiments III and IV - replicas of experiments I and II.

Product size (180 bp) indicated by arrow

3. a. 3. Nested PCR for detection of human DNA

The reasons for developing a nested PCR for the detection of human DNA were:

1. Herpesviral DNA is commonly found associated *in vivo* with cellular DNA, a sensitive human PCR would allow the cell-load of any clinical sample to be determined and the herpesvirus genome titre in that sample calculated as a function of cell concentration.

2. The use of a human PCR allows confirmation of the extraction of DNA from any clinical material and can indicate the presence of PCR inhibitors which may be present following certain extraction techniques. Inhibitors such as haemoglobin and heparin, remaining in blood extractions, have previously been known to result in the generation of "false-negative" DNA detection results (Gelfand and White, 1990).

3. a. 3. i). selection of human DNA PCR primer sequences

First round primers (PDG1 and PDG2) were already available for amplification of a sequence within the human single-copy gene pyruvate dehydrogenase (Koike, 1990) . Use of the DNA sequence database PC Gene provided the genome sequence between the two existing outer primers and allowed design of two internal primers (PDG 3 and PDG 4). As well as their use in a nested PCR for detection of human genomic DNA the second round primers were chosen to span a site of messenger RNA (mRNA). Therefore the size of the second round PCR product would differ depending on whether the input analyte was DNA, or RNA which had been used to generate complementary DNA (cDNA). The use of this PCR also provided a control for DNA contamination in any cDNA synthesis.

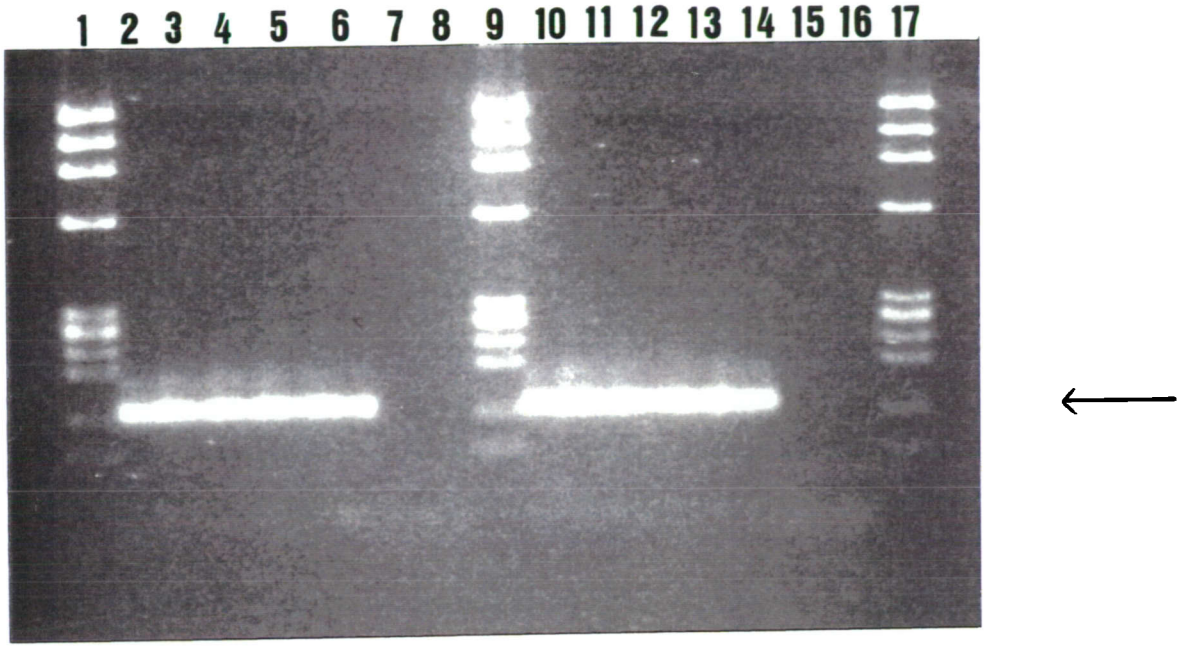
3. a. 3. ii). optimisation of human DNA PCR reaction conditions

As for the HHV8 PCR assay the size of first and second round amplicons of the human PCR, and primer Tm, were similar to that of the PCR for detection of CMV DNA (table 2.1). Therefore the reaction conditions, including primer anneal temperatures, chosen were the same as the CMV PCR reaction. However, when first developed the number of cycles used for amplification was lower, at 30 cycles first round and 20 cycles second round. This number of cycles was, however, found unable to generate PCR product bands of equal, high intensity after the two rounds of amplification. Therefore the cycle number was returned to the standard 35 first round and 25 second round, at this level of amplification equal intensity bands of the predicted size (122 bp, see fig. 3.1.4) were seen after the two rounds of amplification.

3. a. 3. iii). determination of human PCR sensitivity

Cells from the human diploid T-lymphocyte cell line J Jhan was available and were used to provide human DNA for establishment of the sensitivity of the human PCR. Cells were pelleted (800g for 10 minutes), washed in RPMI and resuspended in PBS. The cell number was found to be 1.8 x 10^6 cells / ml. DNA was extracted from 0.5 ml of these cells by phenol / chloroform extraction. The purified DNA was resuspended in 100µl water and found by spectrophotometry to have a concentration of 0.2 µg /µl . A single human cell is known to contain approximately 10 pg of DNA, therefore this solution corresponded to approximately 20,000 cells-worth DNA per microlitre. Replica analysies were performed on a ten-fold serial dilution series of this DNA solution, 2 are shown in figure 3.1.4., and from these analysies the absolute limit of detection for the human PCR was found to be equivalent to approximately a single cells-worth of DNA.

Figure 3. 1. 4. Human DNA PCR detection of genomic DNA extracted from J Jhan cell line



Lanes 1, 9 and 17: molecular weight marker - ΦX 174 cut with *Hae III*
band sizes in nucleotides (from bottom up) 72, 118, 194, 234, 271, 310, 603

Lanes 2-7 and 10-15: ten-fold dilutions of human DNA extracted from J Jhan cell line

lanes 2 and 10: 30 ng DNA (c. 10,000 J Jhan genomes)

lanes 3 and 11: 3 ng DNA (c. 1,000 J Jhan genomes)

lanes 4 and 12: 300 pg DNA (c. 100 J Jhan genomes)

lanes 5 and 13: 30 pg DNA (c. 10 J Jhan genomes)

lanes 6 and 14: 3 pg DNA (c. 1 J Jhan genomes)

lanes 7 and 15: 0.3 pg DNA (< 1 J Jhan genome)

lanes 8 and 16: negative water control

Product size (122 bp) indicated by arrow

3. a. 4. Study of HHV8 DNA prevalence in the venous blood and peripheral samples from HIV-infected and uninfected individuals

3. a. 4. i). introduction

This study was undertaken to investigate the association between the presence of HHV8 DNA in the peripheral blood of an individual and a clinical diagnosis of KS. Previously all epidemiological data associating HHV8 with KS had been based upon the PCR analysis of biopsy material recovered directly from KS tissue (Chang *et al.*, 1994, Boshoff *et al.*, 1995, Moore et al, 1995). Using such analysis it was difficult to determine whether HHV8 was a causal agent of KS or merely a "passenger virus" with tropism for transformed KS tissue. By examination of HHV8 DNA in the systemic environment it was hoped to extend the original tissue-based findings and further investigate the potential for a causal relationship between the virus and disease. Samples such as throat washings and faeces were investigated so that potential routes of virus transmission could be examined.

3. a. 4. ii) study design

Blood samples were available from 189 HIV-infected individuals; 173 male and 16 female. Seventy of the 189 had a Center of Disease Control (CDC) defined diagnosis of AIDS (Centers for Disease Control, 1987). Of those 70 with AIDS, 46 also had a current diagnosis of cutaneous KS. Of the 119 individuals who were HIV-infected but without AIDS 98 did not have any HIV-associated disease, 16 had a non-AIDS disease and 5 were unclassified. 145 individuals had homosexual contact as their primary HIV-acquisition risk factor, 7 were bisexual, 4 possibly homosexual, for 21 a different acquisition route was recorded, and for 12 the route of transmission was unknown. Of the 98 patients with symptom-free HIV infection 77 were enrolled in the 1990

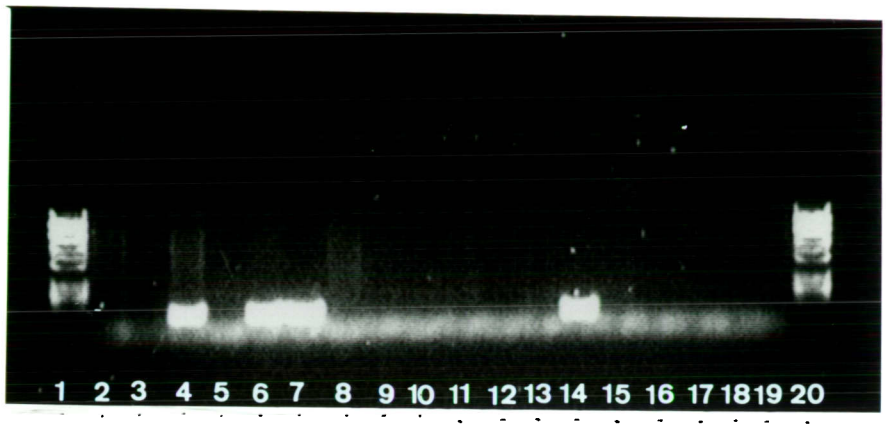
MRC/INSERM Concorde study and these remainder samples, together with others, were used to examine whether presence of HHV8 DNA in the blood of HIV-infected patients without KS was predictive of subsequent KS development. Blood samples were also available from 134 HIV antibody-negative blood donors, these were supplied anonymously by the North London Blood Transfusion Centre. Throat swabs in transport medium were available from 21 AIDS-KS patients and faecal material from 18 such patients. Plasma samples were available from 51 AIDS-KS patients. Serum samples were available from 24 AIDS-KS patients. All patients from whom plasma and serum were tested also had venous blood available for analysis. Samples were prepared as described in section 2.c., DNA from all the blood was prepared from glycigel-stored samples.

The number of circulating CD4 T-cells in venous blood was measured by flow cytometry by members of the Department of Sexually Transmitted Diseases, UCLMS. Statistical analysis was performed by A. Copas, Department of Sexually Transmitted Diseases, UCLMS. Ethical approval for this study was granted by the Clinical Investigation Panel, The Middlesex Hospital.

3. a. 4. iii) results

None of the 134 blood donors had detectable HHV8 DNA in their peripheral blood. HHV8 DNA was detected in the blood of 24 of the 46 patients with AIDS-KS (52%) (table 3.2.1.). In contrast HHV8 DNA was detected in only 11 (8%) of the 143 HIV-infected patients without KS. In addition to analysis for HHV8 DNA a subset of 123 blood samples were investigated for the presence of EBV, another γ-herpesvirus (table 3.2.2.). In this subset 26 (65%) of the 40 patients with AIDS-KS, 19 (58%) of the 33 HIV-infected non-KS patients and 6 of the 44 blood donors (12%) had detectable systemic EBV DNA. Within this sub-set of patients the association between HHV8 and KS remained high with 23 (58%) of the 40 AIDS-KS patients having detectable

Figure 3.2.1. Detection of HHV8 DNA in blood from HIV-infected patients



Lanes 1 and 20 : molecular weight marker - ϕX 174 cut with *Hae III*
band sizes in nucleotides (from bottom up) 72, 118, 194, 234, 271, 310

Lanes 2, 3, 5 and 8 - 13: samples not containing detectable HHV8 DNA

Lanes 4, 6 and 7: samples containing detectable HHV8 DNA

Lane 14 : +ve control, sample known to contain HHV8 DNA

Lanes 15 and 16: negative controls containing water added with reagent pipettes

Lanes 17 and 18: negative controls containing water added with sample pipettes

(Lane 19, unrelated negative sample)

Table 3. 2. 1. Detection of HHV8 DNA in blood from HIV-infected patients and UK blood donors

Patient group	HHV8 DNA		Total number
	detected	**not detected**	
HIV-infected patients:			
with KS	24 (52%)	22 (48%)	46
without KS	11 (8%)	132 (92%)	143
UK blood donors	0 (0%)	134 (100%)	134

Table 3. 2. 2. Detection of HHV8 and EBV DNA in the blood of a sub-set of 73 HIV-infected patients and 50 UK blood donors

Patient group	HHV8 DNA		EBV DNA	
	detected	**not detected**	**detected**	**not detected**
HIV-infected patients:				
with KS	23 (58%)	17 (42%)	26 (65%)	14 (35%)
without KS	2 (6%)	31 (94%)	19 (58%)	14 (42%)
UK blood donors	0 (0%)	50 (100%)	6 (12%)	44 (88%)

By chi squared test there is no association between having EBV DNA and KS: $p = 0.50$

By chi squared test there is a highly significant association between having HHV8 DNA and KS: $p = < 0.0001$

HHV8 DNA, while only 2 (6%) of 33 HIV-infected patients without KS had detectable HHV8 DNA. As detailed before none of the blood donors had detectable HHV8 DNA.

Overall the percentage of HIV-infected patients with KS, if HHV8 DNA was detected, was 69% while the percentage with KS if HHV8 DNA was not detected was only 14%. For the whole group of HIV-infected patients, there was a statistically significant association between having detectable HHV8 DNA in peripheral venous blood and having cutaneous KS ($p<0.0005$ by χ^2 analysis).

As KS is more common in immunosuppressed HIV-negative patients (iatrogenic KS) than the general population and herpesviruses are known to cause a higher incidence of disease in immunosuppressed patients with low CD4 counts (Penn, 1983 and Pertel *et al.*, 1992) a correlation was sought between ability to detect HHV8 DNA and absolute CD4 count. The median CD4 count of patients with detectable HHV8 DNA was 120 (range 0 - 680), while for those without detectable HHV8 DNA it was 300 (range 0 - 1720). There was a strong statistical association between having detectable HHV8 DNA in blood and a reduced CD4 count ($p=0.007$ by Mann-Whitney test). It was possible, using logistic regression analysis, to control for the effects of CD4 count and following this there was still remained a highly significant association between having detectable HHV8 DNA in peripheral blood and a clinical diagnosis of KS (odds ratio 23 (95% CI 8 - 66).

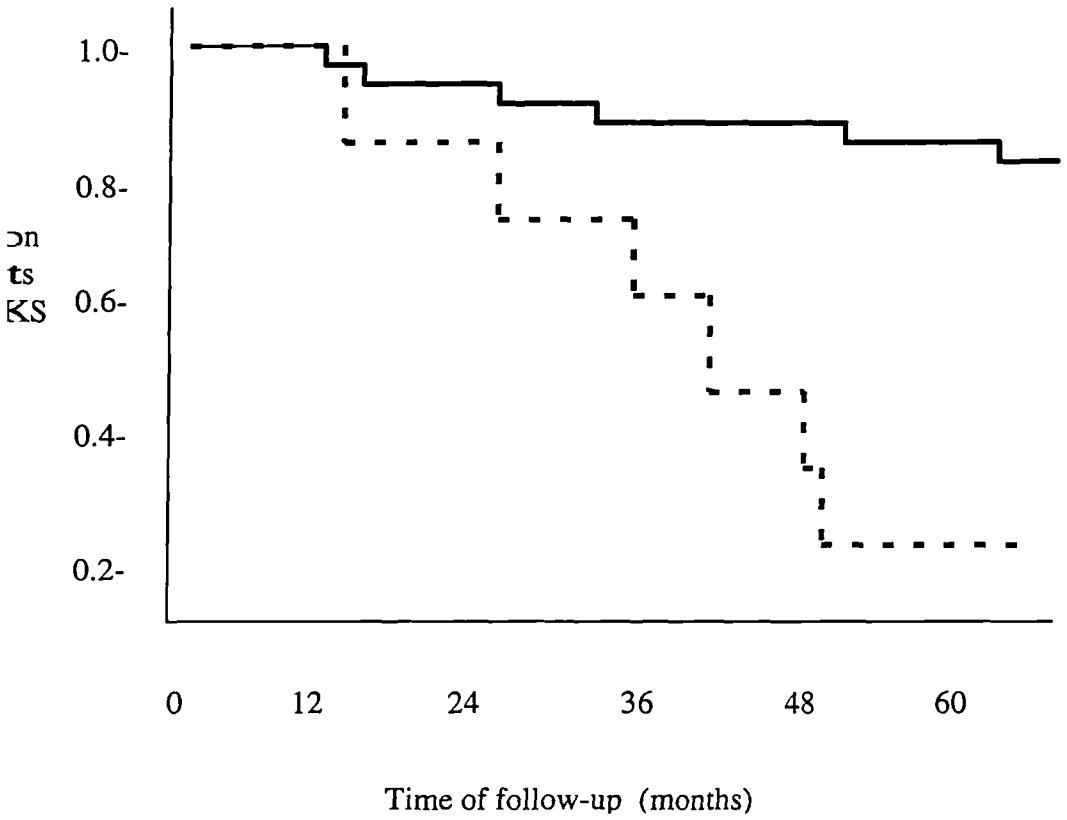
Of the total 189 HIV-infected patients analysed for the presence of systemic HHV8 DNA 143 did not have KS at their first, or only, time of venepuncture. Eleven of the 143 did however have detectable HHV8 DNA in their blood. It was possible, by consultation of patient records, to follow the clinical status of these patients for a median of 30 months (range 0 - 70) after blood sampling. During this follow-up period 18 of the 143 patients developed KS. Six of these 18 patients had been among the group of 11 patients who had detectable

HHV8 DNA in their first sample. In contrast only 12 of the 132 patients without detectable HHV8 DNA at the time of first sampling progressed to develop KS. Patients with more severe HIV-associated disease (CDC group IV disease) had a shorter follow-up (median 2 months, range 0 - 48 months) than those with symptom-free HIV-infection (median 52 months, range 0 - 70 months). From this data the KS-free survival time of all the patients who had HHV8 DNA in their first sample, and of patients who did not, was calculated and represented in a Kaplan-Meier plot (figure 3.2.1.). This plot shows that those HIV-infected patients with detectable HHV8 DNA, but no KS, progressed much more rapidly to develop KS than those who had no detectable HHV8 DNA (log-rank test; $p<0.00005$). Both these two groups had comparable median CD4 counts; 450 for the HHV8 DNA positive patients and 380 for the HHV8 DNA negative patients. It was estimated from the Kaplan-Meier plot that 50% of the patients without KS in the study, who had detectable HHV8 DNA in their blood, developed KS within 3.5 years of their first incidence of HHV8 DNA detection.

In an attempt to identify possible transmission routes for HHV8 a number of peripheral samples were examined, all of which came from patients with KS (table 3.2.3.). Of the 21 throat swab medium samples available, all from patients with detectable HHV8 DNA in their blood, only 1 contained HHV8 DNA. This patient had severe pulmonary and palatal KS at the time of sampling and it was not possible to exclude contamination of the fluid with KS tissue, or tissue exudate from either of these two sites. For comparison these 21 samples were also analysed for the presence of CMV, a herpesvirus known to be commonly shed in the oral cavity of HIV-infected patients. CMV DNA was detected in 16 (76%) of the 21 samples.

HHV8 DNA was detected more commonly in serum from AIDS-KS patients (11 / 24, 46%) than in plasma from AIDS-KS patients (5/30., 16%). In the patients analysed all those with detectable HHV8 in plasma or serum also had HHV8 DNA present in their unfractionated blood. Oro-anal contact has been previously suggested as a possible route of transmission for any agent

Figure 3. 2. 1. Kaplan-Meier plot showing that detection of HHV8 DNA in HIV-infected patients without KS is predictive of subsequent KS development



Proportion of individuals in whose blood HHV8 DNA was (- - - - -) and was not (————) detected and who remained free of KS.

Table 3. 2. 3: Detection of HHV8 and CMV DNA in peripheral samples from HIV-infected patients

Sample	Patient KS status	HHV8 DNA detected	CMV DNA detected
Throat swab	KS	1 / 21 (5%)	16 / 21 (76%)
Plasma	KS	5 / 30 (16%)	NT
Serum	KS	11 / 24 (46%)	NT
Stools	KS	0 / 18 (0%)	NT

NT : not tested

associated with KS, we examined 18 stool samples from AIDS-KS patients, none contained detectable HHV8.

3. a. 4. iv). discussion

There were a number of significant findings made in this large study of HHV8 DNA prevalence in HIV-infected patients without and without KS, and in HIV-uninfected controls. Published in 1995 (Whitby *et al.,* 1995) this was the first study to demonstrate that HHV8 could be regularly detected in systemic circulation of patients with KS and that such detection was significantly associated with a diagnosis of KS. This was an important finding which further strengthened speculation that HHV8 is more than a casual passenger-virus in KS-affected tissue.

The specificity of association between HHV8 and KS was underlined by the analysis of EBV DNA prevalence in the sub-set of patients and controls. EBV, another γ-herpesvirus with no suggested association with KS, was present with equal frequency in both groups of HIV-infected patient regardless of KS status. The detection of EBV, a virus with approximately 50% seroprevalence in the UK population, in blood donors contrasted completely with our lack of detection of HHV8 in any of these individuals. The epidemiological data surrounding KS suggests that any virus associated with KS would be extremely rare in the general population (Beral *et al.,* 1990), the absence of HHV8 DNA in this blood donor population fits well with this epidemiological profile.

Although KS maybe one of the first AIDS-defining illnesses a patient with HIV-infection may experience it can occur throughout the period of AIDS. It had been suggested that the development of KS, and the ability to detect HHV8 DNA in KS lesions, was simply due to the severity of HIV-associated immunosuppression KS patients were experiencing (discussed in Whitby *et al.,*

1995). It was thought that not only did this chronic immunosuppression allow for the formation of KS lesions but it also reduced the host immunological control allowing replication of latent, ubiquitous viruses (such as the herpesviruses, and therefore HHV8). However these results indicate that when the effect of immune suppression is removed by logistic regression analysis there was still an overwhelming association between the detection of HHV8 DNA and KS disease. This suggests that the immunosuppression associated with HIV infection only becomes a risk factor for KS development when HHV8 is also present.

Probably the most significant finding of the study arose from the predictive data showing that as well as the presence of HHV8 DNA being associated with a concurrent diagnosis of KS, that patients without KS who have detectable HHV8 DNA in their blood are far more likely to eventually develop KS. 50% of such patients in this study developed KS within three and a half years of their first HHV8 DNA-positive sample. The finding that having detectable HHV8 DNA is such a strong predictor of subsequent KS development supports the theory of a causal relationship between HHV8 and KS.

Any infectious agent for KS is likely to be restricted to certain groups such as gay men, with transmission likely to be sexual rather than casual. Casual transmission of other herpesviruses such as HHV6, CMV and EBV is usually by contact with infected oral secretions. The finding that HHV8 DNA was detected in only 1 of 21 oral samples, whereas CMV DNA was present in 16 of 21, suggests that HHV8 is unlikely to be transmitted by this route. Another possible route of transmission for a KS-agent was faecal-oral. Studies have found that gay men who experienced high levels of oro-anal contact were at increased of developing KS during the course of their HIV disease (Beral *et al.*, 1993). HHV8 DNA was not detected in any of the stool samples from KS patients, however the presence of PCR inhibitors in this DNA was not excluded prior to analysis. Such inhibitors have been described (Shieh *et al.*, 1995). The presence of inhibitors, notably heparin, may also explain the

disparity in detection of HHV8 DNA between plasma and serum (Saiki, 1989 and Gilchrist *et al.,* 1997). The plasma used in the study was derived from heparin-treated blood, and although the DNA extracted from that blood would have had the heparin removed, DNA in the plasma was not fully purified but simply boiled. In contrast the serum was prepared from unpreserved blood and a correspondingly higher percentage of these serum samples were found to contain HHV8 DNA.

In conclusion the data presented shows that HHV8 has many of the features that a KS-associated agent must possess; from its highly localised prevalence in patients with, or at greatest risk of, KS and its apparent absence in a large blood donor population, to its limited casual shedding. The strong predictive association between presence of the virus in disease-free HIV-infected individuals and subsequent KS disease development is powerful evidence linking virus and disease.

3. a. 5. Association of HHV8 DNA in bronchoalveolar lavage fluid with pulmonary KS

3. a. 5. i) introduction

Although the skin is the most frequent site of KS presentation, mucous membrane, lymph node and visceral organ involvement also occurs (Ioachim *et al.*, 1995). KS at these sites is usually, although not always, in association with cutaneous lesions and systemic disease progression may be rapid. The highest levels of patient morbidity and mortality in individuals with KS occurs when the tumour affects the lungs; pulmonary KS (Pitchenick *et al.*, 1985). Studies have found that between 18% and 32% of patients with cutaneous KS develop pulmonary KS, with a prevalence as high as 47% described at post-mortem (Zibrak *et al.*, 1986, Garay *et al.*, 1987 and Meduri *et al.*, 1986). It is thought that up to 30% of individuals with pulmonary KS will die of respiratory failure as a direct consequence of tumour involvement in the lungs (Mayaud *et al.*, 1990). In a small study of HIV-infected patients who underwent post-mortem at UCL Hospitals 4 of 16 had KS described as a major cause of death and each of these 4 individuals had significant KS involvement of the lungs. This post-mortem study also showed the extent to which KS may affect tissues in the chest cavity with the visceral pleura, mediastinal lymph nodes, lung parenchyma and all parts of the tracheobronchial tree involved.

Currently fibreoptic bronchoscopy is most often used to assist diagnosis of pulmonary KS, by visualisation of "cherry red" or violaceous raised lesions within the trachea and bronchi (Pitchennick *et al.*, 1985). Radiological investigation and the presence of symptoms such as dypsnea, fever and cough are unreliable as confusion is common with other types of infection (Kaplan *et al.*, 1988). Bronchial and transbronchial biopsy are seldom used procedures because of their low diagnostic yield and the associated risk of pulmonary haemorrhage (Zibrak *et al.*, 1986).

In this section a case control study is described which was set up to determine the association between the detection of HHV8 DNA in bronchoalveolar lavage (BAL) fluid and the bronchoscopic visualisation of pulmonary KS. Such detection might be a useful confirmatory diagnostic test for patients with pulmonary KS and may aid the differential diagnosis in patients with lung-cavity KS lesions which were unusual in presentation or difficult to visualise by bronchoscopy.

3. a. 5. ii) study design

In total 82 HIV-infected individuals admitted to the AIDS Unit, UCL Hospitals, for investigation of lower respiratory tract disease were prospectively examined by bronchoscopy and had BAL fluid recovered. Forty-one study patients (40 male) had cutaneous KS, of whom 29 had concurrent pulmonary KS diagnosed by bronchoscopy. Other respiratory tract disease in these 41 patients is detailed in table 3.3.1.The other forty-one patients from whom BAL fluid was available had no clinical evidence of KS, pulmonary or cutaneous. BAL fluid had been recovered from this second group of patients for analysis of other lower respiratory tract diseases; *Pneumocystis carinii* (n=27), bacterial pneumonia (n=10), tracheitis (n=1), *Mycobacterium tuberculosis* (n=2) and *Mycobacterium avium-intracellulare* with *Aspergillus fumigatus* (n=1). Patients were selected for the study on a sequential basis. The median CD4 counts in those patients with and without KS were similar; 30 (range = 0 - 310) and 30 (range = 0 - 330) respectively.

All BAL fluid samples were screened for HHV8 DNA by analysis of 10µl and 50 µl of whole fluid. For analysis of 50 µl the first round volume of the PCR reaction was increased to 100 µl. If a BAL sample was found to contain detectable HHV8 DNA the viral DNA load was determined by 10-fold end-point titration of the BAL fluid. The cell-load of each BAL sample was also calculated by end-point titration PCR for the single copy human gene pyruvate

Table: 3. 3. 1. Results of bronchoscopy and HHV8 DNA detection in BAL fluid from 41 HIV infected persons with KS

Patient No.	Bronchoscopic diagnosis	Bronchoscopic extent of Kaposi's sarcoma		Human DNA (copies/μL of BAL fluid)	HHV8 DNA (copies/1000 cell genomes in BAL fluid)
		widespread/localised	No of visible lesions		
Nos. 1 - 29: Pulmonary and Cutaneous Kaposi's sarcoma					
1	PKS	W	13	100	10
2	PKS	L	2	100	1
3	PKS & *H influenzae* pneumonia	W	18	10,000	1
4	PKS & PCP*	L	3	1000	1000
5	PKS*	W	21	1000	0.2
6	PKS	W	10	1000	0.1
7	PKS & *P aeruginosa* pneumonia*	W	14	1000	0.1
8	PKS & *aeruginosa* pneumonia	W	20	100	10
9	PKS*	W	14	1000	0.02
10	PKS & CMV pneumonitis	W	13	100	0.2
11	PKS & C *neoformans* pneumonia	W	10	1000	0.1
12	PKS & C *neoformans* pneumonia	W	9	10	ND
13	PKS & PCP	W	20	1000	1
14	PKS & PCP*	W	3	1000	0.1
15	PKS	W	5	100	0.02
16	PKS & *M avium intracellulare*	W	2	10	0.02
17	PKS	W	8	1000	1
18	PKS & *M xenopi*	L	8	100	ND
19	PKS	W	9	100	1
20	PKS & P *aeruginosa* pneumonia*	W	10	10,000	1
21	PKS	W	20	1000	1
22	PKS	W	28	1000	10
23	PKS	W	13	1000	0.1
24	PKS	W	9	100	ND
25	PKS	W	32	1000	1
26	PKS & PCP	W	30	10	ND
27	PKS	W	14	1000	ND
28	PKS & PCP	L	6	100	1
29	PKS	L	2	10	10

Table 3. 3. 1. (continued) Results of bronchoscopy and HHV8 DNA detection in BAL fluid from 41 HIV infected persons with KS

Patient No	Bronchoscopic diagnosis	Bronchoscopic extent of Kaposi's sarcoma		Human DNA (copies/μL of BAL fluid)	HHV8 DNA (copies/1000 human genomes in BAL fluid)
		widespread/localised	No of visible lesions		
Nos. 30 - 41: Cutaneous Kaposi's sarcoma only					
30	Mixed bacteria & *MAI**	None	0	1000	ND
31	PCP	None	0	100	ND
32	PCP	None	0	1000	ND
33	*K pneumoniae* pneumonia	None	0	1000	ND
34	*E coli* pneumonia	None	0	100	ND
35	*P aeruginosa* pneumonia	None	0	1000	ND
36	*S pneumoniae* pneumonia	None	0	100	ND
37	PCP	None	0	100	ND
38	PCP	None	0	1000	ND
39	Mixed bacteria & *MAI*	None	0	10,000	ND
40	*Mycobacterium tuberculosis*	None	0	100	ND
41	*Mycobacterium tuberculosis*	None	0	100	ND

Key: PKS = Pulmonary Kaposi's sarcoma; PCP = *Pneumocystis carinii* pneumonia; W = Widespread; L = Localised, ND = Not detected; MAI = *Myocabacterium avium intracellulare*, * = Previously received chemotherapy

dehydrogenase. Neat BAL fluid was also examined using the human PCR to determine whether any samples were inhibitory at that concentration. Where sufficient fresh BAL fluid was available (from 5 patients) CD19, CD4, CD8 and CD14 cell fractions were isolated for analysis and cell-free virus was prepared by ultracentrifugation (cell separation and DNA preparation from these 5 samples was performed by Denise Whitby, Institute of Cancer Research). Blood samples preserved in glycigel were available from a portion of patients. These were extracted as described in section 2. c. Titres of HHV8 DNA in blood were determined as for the HHV8 DNA titres in BAL fluid.

3. a. 5. iii) results

HHV8 DNA was detected in the BAL fluid from 24 (83%) of the 29 individuals with cutaneous and pulmonary KS, but was not detected in BAL fluid from any of the 12 individuals with cutaneous KS only (table 3.3.2.). HHV8 DNA was also undetectable in the BAL fluid from any of the 41 individuals with no KS. This association between detection of HHV8 DNA in BAL fluid and a bronchoscopic diagnosis of tracheobronchial KS was found to be highly significant ($P < 0.001$, two-tailed Fisher's exact test). In 5 of the 24 BAL samples in which HHV8 DNA was detectable it was only detectable when 50 µl was analysed. The analysis of this increased volume was important to the overall sensitivity of the test, raising it from 66% to 83%. Significantly raising the input volume to 50 µl did not result in detection of HHV8 DNA in any of the BAL samples from patients without pulmonary KS.

All the BAL samples analysed contained detectable human genome, indicating the presence of cellular material and no samples were found to be inhibitory.

Similar levels of human DNA were present in BAL fluid from individuals with and without KS. For both groups the median human DNA load was 100 copies per microlitre (range 10 - 10,000 copies per

Table 3. 3. 2. Detection of HHV8 DNA in BAL fluid and peripheral blood of 82 HIV-infected persons with, and without, KS.

	BAL		Blood	
Patient group	**HHV8 DNA detected (%)**	**Range (median) HHV8 copies per 1000 cell genomes**	**HHV8 DNA detected (%)**	**Range (median) HHV8 copies per ml**
KS (n = 41)				
pulmonary & cutaneous KS (n=29)	24 / 29 (83)	0.02 - 1000 (1.0)	17 / 26 (65)	2 - 40 x 10^6 (200)
cutaneous KS only (n = 12)	0 / 12 (0)	-	5 / 7 (71)	2 - 40 x 10^5 (2000)
no KS (n = 41)	0 / 41 (0)	-	0 / 6 (0)	-

microlitre). When levels of HHV8 DNA were calculated as a function of human genome the ratio of virus / human genome was found to vary over a 5 $\log_{10}$ range. No correlation was found between the HHV8 DNA load per human genome and the bronchoscopic extent of pulmonary KS, in terms of number of lesions visible and their widespread or localised nature (table 3.3.1.).

Samples of blood were available from a proportion of individuals with pulmonary KS (26 of 29), cutaneous KS only (7 of 12) and no KS (6 of 41). HHV8 DNA was only detected in the blood of patients with KS and was present at similar prevalences in those with pulmonary KS (17 / 26, 65%) and those with cutaneous KS only (5 / 7, 71%). The median HHV8 DNA titre was similar in the two groups; 200 copies per ml (range 40 - 2 x 10^6 copies per ml) in those with pulmonary KS and 2000 copies per ml (range 40 - 2 x 10^5 copies per ml) in those with cutaneous KS only.

The cell populations were examined from the 5 BAL fluid samples in which cell fractionation was possible. It was found that HHV8 DNA could not be detected in any of the CD4 / CD8 (T lymphocytes) fractions nor the CD19 (B lymphocyte) fraction, but was detected in the CD14 (macrophage) fraction in one sample and in the CD4 / 8 / 14 / 19 depleted cell fractions of all 5 samples. HHV8 DNA was also detected in all the cell-free fractions indicating the presence of cell-free viral particles.

As well as the prospective analysis of BAL fluid samples there was access to a number of samples which had been stored previously. It was therefore possible to analyse BAL fluids taken from patients who in the prospective study had pulmonary KS, but at the time of previous BAL had no bronchoscopic evidence of pulmonary KS (table 3.3.3.). Archive samples from 7 patients were available, taken between 2 and 25 months prior to the time of their first diagnosis of pulmonary KS. At those times 4 of the 7 patients already had cutaneous KS, while 3 had no KS at all. Five of the 7 retrospective BAL fluid samples contained detectable HHV8 DNA, 3 from patients with cutaneous

Table 3. 3. 3. Retrospective analysis in 7 patients of BAL fluid taken before and after development of pulmonary KS.

Patient	KS status at 1st BAL	HHV8 DNA in BAL fluid		Interval between BAL's (months)
		pre-pKS diagnosis	at pKS diagnosis	
1.	cutaneous only	+ ve	+ ve	9
2.	cutaneous only	+ ve	+ ve	16
3.	none	+ ve	+ ve	3
4.	cutaneous only	- ve	+ ve	2
5.	cutaneous only	+ ve	+ ve	6
6.	none	+ ve	+ ve	5
7.	none	- ve	- ve	25

pKS: pulmonary KS

KS, 2 from patients with no KS. In one patient HHV8 DNA was not detected in either the retrospective sample or the sample taken at time of diagnosis of pulmonary KS. The period prior to diagnosis of pulmonary KS in which HHV8 DNA could be detected in BAL fluid ranged from between 2 and 16 months.

3. a. 5. iv). discussion

The previous study in section 3. a. 4. described the association between presence of systemic HHV8 DNA and a diagnosis of cutaneous KS. In this study the association between virus and disease is extended to the pulmonary form of KS. There was a very clear and exclusive association between the bronchoscopic finding of pulmonary KS and the presence of HHV8 DNA in BAL fluid, although virus load did not always correlate with disease extent. The virus was not found in any patients without a diagnosis of pulmonary KS, however in the retrospective analysis the presence of HHV8 DNA in BAL fluid taken from patients prior to their developing pulmonary KS was shown to be a predictor of future pulmonary KS development. It was unfortunately impossible to determine whether any of the patients without HHV8 DNA in their BAL in the prospective study subsequently developed pulmonary KS in the absence of HHV8 DNA.

HHV8, like the other human herpesviruses, is likely to be highly cell-associated. It was for this reason that the level of cellular material recovered in the BAL fluid for all patients was measured. Had the amount of human DNA detected in BAL fluid from patients without pulmonary KS been significantly less than in the BAL fluid of patients with pulmonary KS then the failure to detect HHV8 DNA in patients with no pulmonary KS may have been due to insufficient cellular recovery. However equivalent levels of human DNA were found eliminating this possibility. Important too was the finding that in patients with cutaneous KS only, none of whom had detectable HHV8 DNA in their BAL fluid, the prevalence and titre of HHV8 DNA in blood was similar to that

in the blood of those patients with pulmonary KS. This suggests that the HHV8 DNA detected in the BAL fluid of patients with pulmonary KS is unlikely to have originated from blood contamination of the BAL fluid nor by the non-selective entry of HHV8 DNA-containing blood leucocytes into the bronchial tree. If either of these factors were the processes by which the BAL fluid of a high proportion of the pulmonary KS group came to contain detectable HHV8 DNA then the frequency of viral DNA in BAL fluid should have been similar in both the KS groups.

The sensitivity of detecting HHV8 DNA in BAL fluid from patients with pulmonary KS was increased from 66% to 83% by increasing the volume of BAL fluid analysed from 10 to 50 µl. Such an increase did not however result in the detection of HHV8 DNA in any of the non-pulmonary KS patients. Raising the volume of material analysed increases the risk of introducing greater levels of any PCR inhibitors present, however human DNA was detectable in all samples. It would appear that like CSF samples whole BAL fluid is rarely inhibitory to the PCR assay.

In contrast to previous studies (Moore *et al.*, 1996) which indicated that HHV8 DNA may be detected in circulating B-lymphocytes it was only possible to detect HHV8 DNA in the macrophage fraction of a fractionated BAL sample. Whether the macrophages were active in supporting HHV8 replication or the detection simply reflected their scavenging function could not be determined. It has been previously reported that HHV8 DNA can be detected in the macrophage component of KS lesions (Blasig *et al.*, 1996). It was also interesting to note that in all 5 fractionated BAL fluid samples the cells remaining after depletion contained HHV8 DNA, suggesting that the major cell, or cell-types, which do harbour and / or support HHV8 in the respiratory tract remain to be identified.

Overall these results indicate that there is a significant role for the detection of HHV8 DNA in BAL fluid from all HIV-infected patients suspected of having

pulmonary KS, especially in patients with multiple lung pathology. Analysis of BAL fluid, for the presence of HHV8 DNA, from those patients without current pulmonary KS, but at risk of its development, may also be a useful predictive diagnostic marker. Concurrent with the publication of this study (Howard *et al.*, 1998) Tamm and colleagues (Tamm *et al.*, 1998) published results from a similar study of HHV8 DNA detection in BAL fluid from patients with and without pulmonary KS. They found that HHV8 DNA could be detected in all four prospectively analysed patients with a bronchoscopic diagnosis of pulmonary KS and in all 10 patients with pulmonary KS from whom stored samples were available. In 3 stored BAL samples HHV8 DNA was detected in patients who at that time had no pulmonary KS, however all three of these patients developed pulmonary KS thereafter. From some of the patients multiple samples were available and overall HHV8 DNA was detected in 15 of 19 BAL samples from patients with pulmonary KS. This detection sensitivity, of 79%, is in close agreement with that found in our study, as is the predictive association between having HHV8 DNA in BAL fluid and the subsequent development of pulmonary KS.

3. a. 6. Association of HHV8 DNA in cerebrospinal fluid (CSF) with neurological disease in HIV-infected patients

3. a .6. i). introduction

A number of herpesvirus infections have been associated with severe neurological disease in HIV-infected patients. Cytomegalovirus has been found at post-mortem in approximately one-third of AIDS patients and is associated with encephalitis, polyradiculopathy and myelitis (Morgello *et al.*, 1987). Epstein-Barr virus has been detected in almost all primary CNS lymphomas (MacMahon *et al*, 1991 and Pedneault *et al.*, 1992). The detection of both CMV and EBV infection in the neurological setting has usually been by the use of PCR on either tissue or CSF samples. (Arribas *et al*, 1995 and Cinque *et al.*, 1996).

This study was undertaken to determine the prevalence of HHV8 DNA in CSF from a panel of HIV-infected patients who had presented with neurological disease. Three cases of suspected HHV8-associated encephalitis have been described in which HHV8 DNA was found in material obtained by stereotatic brain biopsy for diagnosis of unexplained encephalopathy (Said *et al.*, 1997), and BCBL's, which are associated with HHV8, have been described in the CNS of HIV-infected patients (Cesarman *et al.*, 1995). However the general incidence of KS in the CNS is low with the largest reported study showing that less than 1% of homosexual HIV-infected patients have evidence of neurological KS when examined at post-mortem (Levy *et al.*, 1985).

3. a. 6. ii) study design

A panel of 36 CSF samples were available from patients who had undergone diagnostic lumbar puncture for investigation of neurological disease. Of the 36 samples available 10 came from patients with evidence of CNS lymphoma (as

shown by the presence of symptoms suggestive and the presence of EBV in CSF and tissue), 4 from patients with suspected neurological CMV disease and 4 from patients with suspected HIV encephalopathy. The 18 other patients had an alternate neurological diagnosis. Seven of the patients also had cutaneous KS. The median CD4 count in this patient group was 30 (range 0 - 350).

Before storage CSF samples had been separated by centrifugation into their cell and supernatant fractions, as described in section 2. c.. The supernatants were boiled before addition to the PCR reaction, DNA from the cell pellets was extracted in 20 µl EB (Appendix A), before boiling and addition to the PCR (see section 2. c.). Ten microlitres of both supernatant and cell pellet preparations were analysed for the presence of HHV8 DNA.

3. a. 6. iii). results

HHV8 DNA was not detected in any of the 36 CSF supernatants but was detected in 2 of the 36 (6%) CSF pellets. One of these CSF's came from a patient with systemic, but not CNS lymphoma and no clinically apparent KS This patient subsequently developed CNS lymphoma over a year after the recovery of CSF. The other CSF came from a patient with disseminated CMV disease, no CNS lymphoma, but widespread cutaneous KS. Both these patients also had HHV8 DNA in their blood, at 2×10^5 and 2×10^6 copies per ml respectively. The presence of systemic HHV8 DNA was undetermined for the other patients in the study. There was no evidence of leucocytes or pleocytosis in either of the two CSF samples in which HHV8 was detected.

By comparison EBV DNA was detected in the CSF of all 10 patients with brain-biopsy-confirmed CNS lymphoma and in 8 (31%) of the the 26 CSF samples from patients without known CNS lymphoma.

3. a. 6. iv) discussion

Overall the prevalence of HHV8 DNA (6%) in the CSF samples from this group of extremely immunosuppressed HIV-infected patients was low, with no specific association found between any neurological condition and the presence of HHV8 DNA in the CNS. This compares with the much higher prevalence of EBV DNA, which was detected in the CSF of all patients with suspected CNS lymphoma, and in over 30% of patients without a specific EBV-associated neurological disease. It is possible that the HHV8 DNA detected in the 2 CSF samples was derived from blood contaminating the samples during lumbar puncture, or from the import of immunologicaly-important leucocytes into the CSF, which has been reported (de Gans *et al.*, 1990). Both patients did have HHV8 DNA present in their blood, however in both cases no white blood cells were noted after microscopic examination of the CSF, nor where the samples reported to have been grossly contaminated during recovery.

Although this study had access to a limited number of CSF samples it would appear that unlike a number of the other herpesviruses, such as CMV and EBV which have been found commonly associated with CNS disease in extremely immunosuppressed HIV-infected patients (Morgello *et al.*, 1987 and Pednanultt *et al.*, 1992), HHV8 infection is uncommon in the neurological environment of this patient group.

3. a. 7. HHV8 DNA prevalence in the semen of HIV-infected homosexuals and UK semen donors

3. a. 7. i) introduction

The incidence of KS in the HIV-infected population suggests that it may be caused by a sexually transmitted infectious agent (Beral *et al.,* 1990). Such an agent, which is likely to be rare in most western populations, should also be prevalent in individuals at increased risk of developing KS, such as gay men. Recently it has been shown that KS is more likely to occur in men with a high number of homosexual partners and that rates of seropositivity for HHV8 are associated with increasing numbers of homosexual sexual contacts (Martin *et al.,* 1998 and Melbye *et al.,* 1997). Such data suggests that if HHV8 is the causative agent of KS then the virus may well be transmitted via infected semen and that such transmission may be more common in the homosexual population than the heterosexual population.

This study was conducted to determine the prevalence of HHV8 DNA in the semen of both HIV-infected gay men and heterosexual semen donors from the UK. It sought to examine whether the virus could be found in semen, and therefore be sexually transmitted, and, if found in semen, there was an association between sexual orientation, immunosuppression and viral genome prevalence.

3. a. 7. ii) study design

Twenty-four HIV-1 infected gay men were recruited from a London HIV outpatient clinic, each providing at least one semen sample for analysis. As well as providing semen samples blood was taken from each by venepuncture. Seventeen of these men had AIDS, 15 with KS and 2 without KS. Of the remainder 6 were symptom-free and one had non-AIDS CDC group IV

disease. Cryopreserved semen samples were also available from 115 men who had donated semen at a London fertility clinic over the last 15 years. HIV-infected patients providing samples gave informed consent and the study was approved by the Camden and Islington Community Health Services Trust Ethics Committee.

Semen samples were processed for DNA extraction within 4 hours of production by colleagues at the The Institute of Cancer Research, London. The semen was separated into cell and seminal plasma (cell-free) fractions which were then extracted separately by the phenol chloroform technique (section 2. c. 4.). Semen from the healthy donors, which had been cryopreserved, was thawed and extracted by myself in the same way as fresh semen. Blood samples were stored and the DNA extracted using the QIAGEN method detailed in section 2. c. 1. In the study PCR assays for the detection of human, CMV and HHV8 DNA were performed as described in section 2. b. The presence of CMV DNA in the semen samples was investigated as CMV has previously been detected in the semen of both HIV-infected and uninfected individuals and sexual transmission of CMV, especially between gay men is well documented. (Drew *et al,* 1984 and Tijam *et al.,* 1987). The PCR for detection of human DNA was used to determine the levels of human DNA recovered from the semen samples of 40 donors and all 24 HIV-infected homosexuals.

Seminal fluid has been found to be a significant inhibitor of the PCR process (Dyer *et al.,* 1996). To confirm that the DNA prepared from these samples was not inhibitory to the PCR assays a ten-fold dilution series of purified HHV8 DNA was prepared in an HHV8 DNA-negative semen sample and the DNA purified as previously described. The HHV8 PCR detection sensitivity in these samples was then compared with the same HHV8 DNA dilution series diluted in distilled water. To confirm that cryopreservation and subsequent thawing of the donor semen did not adversely affect DNA yield or introduce PCR inhibitors known cell numbers of the HHV8 genome-containing cell line, HBL-6 (20 - 50 copies HHV8 DNA per cell) were introduced into an HHV8

DNA-negative sample and the sample prepared for cryopreservation. After overnight storage in liquid nitrogen the sample was thawed and processed as usual. The titre of HHV8 DNA recovered was then assayed and compared to the titre of HHV8 DNA in the same number of HBL-6 cells suspended in RPMI (appendix A) which had been extracted immediately.

3. a .7. iii) results

The extracted DNA from seminal fluid was found to have no inhibitory effects on the sensitivity of the HHV8 PCR and the cryopreservation procedure resulted in no loss in DNA yield or assay sensitivity. The median level of human DNA recovered from the semen was calculated, by serial end-point titration, to be the same for both the HIV-infected gay men and the heterosexual donors, at 1 x 10^5 copies per microlitre.

Semen from 6 (25%) of the 24 HIV-infected gay men contained detectable HHV8 DNA, 20 (83%) of 24 contained detectable CMV DNA (table 3.4.1.). Three samples contained both genomes. HHV8 DNA was detected in 3 of the 15 patients with KS (patients 10, 11 and 15, table 3.4.2.) and in 3 of the 9 patients without KS (patients 19, 22 and 24). HHV8 DNA was found most often in the seminal plasma (5 of 6 patients). One patient had HHV8 DNA in both the seminal plasma and cellular fractions (patient 10) and one patient had HHV8 DNA in the cellular fraction alone (patient 15). The median CD4 count in the patients with detectable HHV8 DNA in semen was 220 (range 90 - 740) and in those without was 100 (range 10 - 490).

Four of the 6 patients with HHV8 DNA in semen also had detectable HHV8 DNA in their blood. Overall HHV8 DNA was detected in the blood of 10 / 15 (67%) of the HIV-infected patients with KS, and both the HIV-infected patients without KS, who had HHV8 DNA in their semen, also had detectable HHV8 DNA in their blood.

Table 3. 4. 1. Detection rates for HHV-8 DNA and CMV DNA in semen and blood from HIV-infected individuals, and in semen from UK semen donors.

	Number tested	HHV-8 DNA in semen (%)	HHV-8 DNA in blood (%)	CMV DNA in semen (%)
Semen donors	115	0 / 115 (0)	nt	4 / 115 (3)
HIV-infected gay men:				
with KS:	15	3 / 15 (20)	10 / 15 (67)	12 / 15 (80)
without KS:	9	3 / 9 (33)	2 / 9 (22)	8 / 9 (89)

nt : not tested

When analysed by Fishers exact test there is no statistical significance, at the 0.05 level, between the prevalence of HHV8 DNA in the semen of HIV-infected gay men with and without KS; (P = 0.1)

Table 3. 4. 2. Sites of HHV-8 DNA detection in HIV-infected individuals with, and without* KS who had detectable HHV-8 DNA in either semen or blood.

	Blood	Semen
Patient 2	**+ve**	-ve
2 rpt	nt	-ve
Patient 5	**+ve**	-ve
Patient 7	**+ve**	-ve
Patient 10	**+ve**	**+ve** [1]
10 rpt	nt	**+ve** [1]
10 rpt	nt	**+ve** [2]
Patient 11	**+ve**	**+ve** [2]
Patient 12	**+ve**	-ve
Patient 14	**+ve**	-ve
Patient 15	-ve	**+ve** [3]
Patient 16	**+ve**	-ve
Patient 19*	**+ve**	**+ve** [2]
Patient 20	**+ve**	-ve
Patient 21	**+ve**	-ve
Patient 22*	**+ve**	**+ve** [2]
Patient 24*	-ve	**+ve** [2]

nt: not tested

rpt: repeat sample

[1] HHV-8 DNA in both cell and plasma fractions

[2] HHV-8 DNA in plasma fraction alone

[3] HHV-8 DNA in cellular fraction alone

In contrast HHV8 DNA was not detected in any of the semen from the 115 healthy donors; CMV DNA was detected in the semen of 4 of these healthy donors. HHV8 DNA was still not detected in any of the 115 donor semen when ten-fold more DNA was then re-analysed from each sample.

3. a. 7. iv) discussion

In this study HHV8 DNA was not found in 115 heterosexual semen samples donated for use in assisted conception over the past 15 years. This finding is in concordance with data from section 3. a. 4. in which HHV8 DNA could not be detected in the blood of a large panel of blood donors. It suggests that HHV8 can, in the UK, be rarely found in individuals outside those who are immunosuppressed and from groups known to be at high-risk of developing KS. Furthermore it would seem that, at present, there is a minimal risk of acquiring HHV8 from donated semen in the UK.

Importantly the absence of detectable HHV8 DNA in the semen of healthy HIV-negative individuals contradicts a number of earlier studies from north America and Italy which suggested that HHV8 DNA could be detected in the semen of a significant minority of HIV-negative heterosexuals. (Lin *et al.*, 1995 and Monini *et al.*, 1996). Lin and colleagues originally reported finding HHV8 DNA in the semen of 23% of HIV-negative donors, although recently this data has been withdrawn from publication when their results could not be repeated (Lin *et al.*, 1998). Instead the findings of this study would agree with a number of more recent studies which were unable to find HHV8 DNA in the semen and prostate from a range of different populations of HIV-uninfected men, including those from north America and Italy (Corbellino *et al.*, 1996, Tasaka *et al.*, 1996 and Gupta *et al.*, 1996). As the current estimate of HHV8 seroprevalence in the UK is between 2 and 5% (Simpson *et al.*, 1996) the absence of detectable HHV8 DNA in these UK semen donors would appear unsurprising.

The seroprevalence of CMV in the UK is approximately 50%, however only 4 semen donors had detectable CMV genome in their semen, underlining the difficulty of detecting herpesviral DNA in the semen of individuals with normal immunostatus. Over 80% of the HIV-infected patients (who are all likely to have been previously infected with CMV) had CMV detected in their semen, a level in accordance with a number of previous studies (Drew *et al.,* 1984 and Tijam *et al.,* 1987). This illustrates the emergence of active CMV infection in patients with reduced immunological control. and the relative ease of CMV DNA detection in the semen of such individuals.

Twenty-five percent of the HIV-infected patients had detectable HHV8 DNA in their semen and the virus was detected in a slightly higher percentage of semen from those without KS (33%) than those with KS (20%). Overall this difference was not statistically significant owing to the limited number of patients from whom semen was available. It should be remembered that all the HIV-infected patients included in the study were homosexual, and therefore at risk of developing KS, with 12 / 24 (50%) having detectable HHV8 in their blood. The finding that HHV8 DNA was more commonly detected in seminal plasma was unusual as herpesviruses are predominantly cell-associated.

The HIV-infected patients in whom HHV8 DNA was detected in semen were experiencing, on average, a less severe degree of immunosuppression than those in whom HHV8 DNA was not detected, indeed one patient with detectable HHV8 DNA in semen had a CD 4 count of over 700. Again with limited samples this was not a statistically significant finding, but may be an important observation if transmission of the virus is by sexual contact. For sexual transmission to occur infected individuals would need to remain sexually potent, and therefore relatively healthy in order to engage in activities allowing for viral transmission to others. Such shedding of CMV in the semen of healthy gay men is well described and is likely to contribute to the transmission and high seroprevalence of CMV in the gay community (Lange *et al.,* 1984).

Three of the HIV-infected patients without KS had detectable HHV8 DNA in their semen, 2 of whom also had the virus detected in peripheral blood. It is not yet known whether the finding of HHV8 DNA in semen is associated with future development of KS, however combined with a systemic HHV8 viraemia this would be expected from the findings of the study described in section 3. a. 4.

Even with the limited number of semen samples available from HIV-infected homosexual men, these findings strongly support the proposed sexual transmission of HHV8 within this social group. The presence of HHV8 DNA in the semen of 30% of the gay men tested correlates well with the epidemiology of KS. Studies have shown that 30% of all gay men with HIV-associated immunosuppression will develop KS (Peters *et al.*, 1991). The lack of HHV8 DNA in a large number of semen samples from heterosexual donors would suggest that this virus is both uncommon in the general UK population and difficult to detect in those few heterosexual immunocompetent individuals who are infected. The incidence of HHV8 sexual transmission between heterosexual partners in the UK is therefore likely to be low. There are however two reports in which women have developed KS following sexual contact, both of which occurred with a male partner from a high KS-risk social group (Tirelli *et al.*, 1996 and Barry *et al.*, 1991). These cases must then support the possibility of heterosexual transmission of HHV8 with such transmission potentially involved in the low-level maintenance of HHV8 in the general population. Evidence of heterosexual transmission is also of importance in defining the infectious risk factors associated with artificial assisted conception procedures where semen is used without prior screening to determine the HHV8 serological status of the donor.

3. b. Response of HHV8 to chemotheraputic intervention in HIV-infected patients with KS

3. b. 1. Introduction

The management of AIDS-associated KS is usually directed by the severity of disease and the level of immune-system impairment experienced by the patient. Current treatment protocols are particularly unsatisfactory for patients with cutaneous lesions and no evidence of systemic or lymph-node involvement. In this circumstance the CD4 count of the patient has been proposed as a directing factor. If the CD4 count is above 200 then local treatment with intra-lesional chemotherapy or local radiotherapy has been found effective, however this fails to influence the development of new lesions (reviewed in Volm and Roenn, 1995 and in Tur and Brenner, 1996). Alternatively for such patients systemic anti-retroviral treatment with either zidovudine (AZT), or one of new HIV-1 protease inhibitors has been suggested to facilitate immune system repair. In small studies such therapies have been found to provide clinical stabilization of KS tumour load (Shingardia *et al.*, 1995 and Lebbe *et al.*, 1998). Such anti-retroviral therapy may also be combined with α-interferon treatment, depending on the patients ability to tolerate the associated side-effects of myelosuppression and "flu-like" symptoms. Unfortunately α-interferon treatment is of little benefit in those patients who have had previous systemic chemotherapy or are experiencing viral opportunistic infections (Evens *et al.*, 1991).

In patients with CD4 counts of less than 200 α-interferon therapy is largely ineffective and systemic cytotoxic chemotherapy is required. Previously a number of single-agents and combinations have been evaluated including bleomycin, vincristine and adriamycin (Laubenstein *et al.*, 1984 and Gill *et al.*, 1996). Recently liposomal daunorubicin, an anthracycline derivative delivered in a phospholipid membrane, has been shown to provide a partial tumour

response in between 50 - 60% of patients (Harrison *et al.*, 1995). However as with all systemic cytotoxic chemotherapies side affects are common. For daunorubicin these include including myelosuppression, hepatotoxity, cardiac damage and liver failure. The toxicity of these chemotheraputic regimes, their unsuitability in early disease and the restricted use of other treatments has led to the investigation of a variety of alternative agents. These include systemic retinoids, β-HCG, retinoic acid and sulfated polysaccharide peptidoglycans, all of which have shown some promise in *in vitro* analysis and are undergoing clinical evaluation (Jie *et al.*, 1997, Lang *et al.*, 1997 and Bower *et al.*, 1997). Since the discovery of HHV8 and its proposed involvement in the development of KS anti-herpesviral agents have also been suggested as possible treatments, although data as to the effectiveness of PFA and GCV is limited (Morfeldt and Torssander, 1994 and Kedes and Ganem, 1997).

Because of the hypervascular nature of the KS tumour agents capable of interfering with angiogenesis are also currently being evaluated as potential therapies. One of these, thalidomide, is of particular interest because of its low toxicity profile and the general tolerance shown by HIV-infected patients who have been treated with the drug for pharyngeal ulceration and aphthous stomatitis (Youle *et al.*, 1990). As well as having a potent anti-angiogenic activity thalidomide is also a selective inhibitor of TNF-α synthesis (D'Amato *et al.*, 1994). TNF-α has been shown *in vitro* to stimulate KS spindle cell proliferation and be crucial in the support of cell lines derived from KS tissue (Samaniego *et al.*, 1997). Thalidomide also induces the anti-inflammatory T helper cell type 2 cytokine response while inhibiting the pro-inflammatory T helper cell type 1 response which includes interferon-γ, a cytokine known to stimulate spindle cell transformation and the mitosis of KS cells (Moreira *et al.*, 1993, McHugh *et al.*, 1995 and Fiorelli *et al.*, 1995).

This chapter describes a number of patient reports and a small phase II trial in which a variety of alternative therapies for the treatment of KS have been

examined. In all cases the virological response of HHV8 to these therapies is described along with any macroscopic clinical changes in KS disease extent.

3. b. 2. Clinical and virological response in a child with HIV-associated KS during treatment with AZT.

This case study involves a 3 year old girl of Ugandan origin who presented to Great Ormond Street Hospital for Sick Children in July 1992. At this initial presentation the child was experiencing recurrent respiratory infections in association with generalised lymphadenopathy and hepatosplenomegaly. Laboratory analysis showed her to be HIV-1 infected, the source of infection was presumed to be maternal. At this time her CD4 T-lymphocyte count was 400. Four months after her diagnosis with HIV the girl was commenced on septrin prophylaxis (November 1992) and enrolled in the PENTA-1 trial. The PENTA-1 trial was a double blind placebo controlled trial comparing immediate versus deferred therapy with azidothymidine (AZT), upon unblinding it was discovered that the girl had received AZT while on the trial.

For the next 3 months while receiving AZT the child remained well (February, 1993), however at maternal request she was removed from the PENTA-1 trial. The child continued to be asymptomatic for the following 12 months, although her CD4 count declined to 33. After this 12 month period AZT therapy was recommenced at 360 mg / m^2 / day (January 1994). Seven months later (August 1994) a firm, painless swelling appeared in the left femoral region, this was associated with thigh oedema. The swelling was biopsied 4 months later (December 1994) and an associated lymph node removed. Histological examination of the biopsy tissue showed that almost the entire normal architecture of the lymph node had been replaced by KS tissue. Staining for acid-fast bacilli, fungi and CMV were negative, no viral inclusion bodies were noted. After the diagnosis of lymph node KS the girl was commenced on liposomal daunorubicin at 40 mg / m^2 every 3 weeks for 18 weeks. At this time her CD4 count had declined to 20 and she continued on AZT. Within 1

month of initiation of daunorubicin therapy a clinical response in the femoral swelling was noted with a decrease in oedema, however 3 months after cessation of chemotherapy (May 1995) there was a recurrence of both lymphadenopathy and oedema. In an attempt to relieve the recurrent symptoms a course of localised radiotherapy was administered and the swelling diminished. Two months after this response to radiotherapy multiple cutaneous KS lesions appeared on the limbs (October 1995).

A number of blood samples were available for analysis for the presence of HHV8 DNA. Table 3.5.1. gives a brief summary of the clinical course of this child as well as the therapy received and dates of blood sampling. All blood samples were stored in the glycigel freezing medium and DNA was extracted as described in section 2. c. 1. The first date of venepuncture was November 1992, at the time of enrollment into the PENTA-1 trial. Although this sample was taken over 2 years before the development of lymph node-associated KS HHV8 DNA was detected at a low titre of 200 copies / ml. Such detection is in agreement with the earlier study (section 3. a. 4.) in which HHV8 DNA in blood was found to be predictive of subsequent KS development. Interestingly in the next available blood sample, taken 3 months after initiation of AZT therapy, just prior to removal from the PENTA-1 study, HHV8 DNA could not be detected. It is possible that the 3 month treatment with AZT resulted in an immune-system enhancement allowing clearance of the low level of systemic HHV8 DNA detected 3 months earlier.

Although no blood sample was taken at the time of first persistent groin swelling and histological diagnosis of lymph node KS, biopsy tissue was available for analysis. HHV8 DNA could be detected in the tissue confirming the visual microscopic diagnosis of KS. At this time daunorubicin therapy was commenced. The next time of blood sampling was at the end of the 18 week daunorubicin course and, as after the AZT therapy HHV8 DNA was still undetectable. This may have been the consequence of two effects; firstly the daunorubicin may have had a direct anti-HHV8 effect thereby eliminating detectable HHV8 DNA from the blood, or by reducing the gross tumour load

Table 3. 5. 1. Clinical course, treatment regimen and presence of HHV8 DNA in the blood of a child recieving AZT therapy.

Date	Clinical course	Treatment	HHV8 DNA in blood
7 / 92	HIV diagnosed	-	ns
9 / 92	entered Penta trial	AZT	ns
11 / 92	-	AZT	**+ ve (200 copies / ml)**
2 / 93	removed from Penta-1 trial	-	- ve
1 / 94	declining CD4 count	AZT	ns
12 / 94	femoral lymph node biopsy shows KS	AZT and Daunorubicin	ns **(HHV8 DNA detected in biopsy)**
3 / 95	clinical response of KS	AZT and Daunorubicin	- ve
5 / 95	relapse of KS	AZT and radiotherapy	**+ ve (2000 copies / ml)**
10 / 95	cutaneous KS	AZT	**+ ve (200 copies / ml)**

ns - no sample

in the lymph node (as was noted) there may have been less support for HHV8 replication making its detection in the blood more difficult. At the time of lymph node KS relapse, and reappearance of oedema, HHV8 was once more detectable in peripheral blood at 2000 copies / ml, a 10-fold higher titre than when first detected in November 1992. As this re-emergence of HHV8 corresponded with increase in tumour mass it may be that levels of HHV8 in the blood do respond to changes in tumour bulk. HHV8 DNA remained detectable in the peripheral blood at a level of 200 copies / ml just prior to the development of widespread cutaneous KS in October 1995.

In this study the detection of HHV8 DNA in a lymph node biopsy was used to confirm the histological diagnosis of KS, and levels of HHV8 DNA in blood were found to mirror the use of immunosupportive AZT therapy and the decrease in KS tumor bulk after systemic chemotherapy, as well as the re-emergence of KS following therapy failure. These data suggest that detection, and simple quantification, of HHV8 DNA in peripheral blood may be a useful surrogate marker for KS tumor therapy, response and progression in the HIV-infected patient. As had been previously noted detection of HHV8 DNA in peripheral blood was predictive of eventual KS development.

3. b. 3. Clinical and virological response in a child with HIV-associated KS during treatment with thalidomide

This second case study also involves a young female with maternally acquired HIV infection. This 13 year old girl was of Zairian origin and had emigrated to Switzerland at the age of 9. Until this time she had been clinically well but then had several periods of pneumonia. Over the next 3 years she developed a number of HIV-associated diseases, including recurrent oral candidiasis, lymphocryptic interstitial pneumonitis and PCP. For over 1 year prior to her development of KS, at the age of 13, her CD4 count had been 0. In early 1995 she presented with subcutaneous nodular KS lesions on both arms, the left upper leg and the right eyelid. Diagnosis of KS was made by histological

examination of biopsy material which showed the proliferation of spindle-shaped cells and vascular structures with large endothelial cells, both common characteristics of KS tumour development. After the initial diagnosis no specific chemotherapy or radiotherapy for KS treatment was initiated and the number and size of the lesions increased gradually over a 4 month period.

Approximately 4 months after the first development of KS (at age 14 years, 3 months), the child developed painful oral ulcers for which no causative pathogen could be identified. Attempts to treat this oral ulceration with topical and systemic corticosteroids failed and therapy with thalidomide was started at a dose of 3 mg / kg / day. The initial thalidomide treatment duration was three weeks during which time a coincidental decrease in the size of the original KS lesions was noted, as well as the absence of any new lesions. As both the oral ulcers were responding to treatment and the severity of KS appeared to be decreasing thalidomide treatment was continued. Two months later still no new lesions had developed and the number of existing lesions had decreased from 29 to 13 (at 14 years, 7 months). During this period the treatment with thalidomide was augmented with local radiotherapy to improve resolution of KS lesions at an interpharangeal joint and upper eye lid.

Unfortunately at this time (14 years, 8 months) the girl developed a *Candida albicans* septicemia. This required intravenous therapy with amphotericin B, resulting in severe neutropenia. To counter the neutropenia granulocyte colony-stimulating factor (G-CSF) was administered. The introduction of G-CSF was associated with an increase in the size of the existing KS lesions. With resolution of the *Candida albicans* septicemia G-CSF and amphotericin B therapy was stopped and thalidomide treatment, which had continued throughout, continued. After cessation of G-CSF treatment the extent of the KS tumour bulk again decreased and then remained stable for almost 1 year while the child continued on thalidomide. At this time she died, exact cause of death was not established. Throughout the thalidomide treatment no adverse effects were noted.

Blood and skin biopsy samples were available at various times before and during this child's thalidomide treatment. Table 3.5.2. shows the time at which these samples were taken and the therapies being used at those times, together with HHV8 DNA analysis of blood and biopsy tissue. As well as the qualitative detection of HHV8 DNA in blood and the titration of viral load in biopsy material human DNA load in the extracted biopsy was also measured (as described in section 3. a. 5.). Such quantification enabled the HHV8 DNA load in the biopsy tissue to be calculated as copies per human genome and allowed more accurate comparison between different biopsy samples.

Prior to initiation of thalidomide therapy HHV8 DNA could be detected in the peripheral blood and in biopsies of KS and, for comparison, normal tissue. At this time a very high level of HHV8 DNA; 10 copies per human genome, was detected in the tumor biopsy. As HHV8 DNA could be detected in the blood it was postulated that the detection of HHV8 DNA in normal skin may have been due to contamination of the tissue biopsy with HHV8 DNA-containing blood. Samples taken three months later, after initiation of thalidomide therapy, showed a dramatic decrease in the HHV8 DNA tumour load, and no detectable HHV8 DNA in the peripheral blood or the normal skin biopsy. This finding supports the theory that the original detection of HHV8 DNA in the normal biopsy may have been due to blood contamination. Most significantly the HHV8 DNA load in the tumor tissue, at a time when the KS tumor bulk of individual lesions was noted to be clinically decreasing, had reduced from 10 copies per human genome to 0.1 copies per human genome; a 100-fold reduction. Six days after commencement of G-CSF therapy however HHV8 DNA was once more detectable in peripheral blood, although still none could be detected in the control tissue. At this time-point no tumour tissue was available for analysis. The final blood sample taken was 3 months after G-CSF therapy had been stopped and the patient was once more experiencing stable KS, while continuing to receive thalidomide therapy. At this time HHV8 DNA was again undetectable in the blood. Tissue samples were not available.

Table 3. 5. 2. HHV8 DNA in the blood and tissue of an HIV-infected child before and during treatment with thalidomide.

Age	Therapy	HHV8 DNA in blood	HHV8 DNA copies per human genome in tissue	
			Tumor	**Non-tumor**
14 yrs, 3 mths.	none	**detected**	**10**	**0.001**
14 yrs, 7 mths.	thalidomide	not detected	**0.1**	not detected (< 0.0001)
14 yrs, 8 mths.	thalidomide + G-CSF	**detected**	ns	not detected (< 0.0001)
14 yrs, 11 mths.	thalidomide	not detected	ns	ns

ns: no sample

This study shows both a clinical and virological response of KS and HHV8 DNA load to treatment with thalidomide in a young female with HIV-associated KS. Interestingly upon initiation of G-CSF therapy the clinical benefits of the thalidomide treatment appeared to be counteracted and HHV8 DNA load increased. Both these effects were reversed upon cessation of G-CSF therapy and continuation of thalidomide treatment alone. Possible molecular mechanisms for these effects are as follows; thalidomide is an inhibitor of TNF-α and angiogenesis, while inducing T helper cell type 2 cytokine production and inhibiting T helper cell type 1 cytokine production. These effects act to reduce KS spindle cell formation and mitosis of KS cells. This would explain the reduction of existing KS tumour bulk noted and the absence of new KS tumour development. However G-CSF has an antagonistic effect to thalidomide and stimulates human endothelial cell proliferation (Bussolino *et al.,* 1989), thus aiding the development of new KS tumour bulk as was seen clinically. Since other therapy, in particular anti-retroviral, was unchanged during this period it is likely that the above mechanisms exerted an effect on KS tumor load and the changes in HHV8 DNA load in both tumour and blood. Again it was impossible to determine whether thalidomide was having a direct effect of HHV8 itself or whether the variation in HHV8 DNA load was a response to change in clinical KS tumour bulk.

In summary it would appear that the use of thalidomide in this case proved of definite clinical benefit, in terms of the control and partial resolution of severe cutaneous KS lesions experienced by this girl. HHV8 DNA titre in blood and tissue was again found to mirror the extent of KS tumor load. These findings reinforce the previous suggestion of the use of HHV8 genome load, determined by simple titration quantification, as a surrogate marker of KS response to systemic therapy.

3. b. 4. A phase II study of thalidomide use in HIV-infected patients with early KS

Following the findings, described in section 3. b. 3., of the potential use of thalidomide in the treatment of HIV-associated KS a collaborative study was initiated with colleagues at the Kobler Clinic, Chelsea and Westminster Hospital, London to investigate the use of thalidomide as an alternative therapy to conventional treatments for the management of early HIV-associated cutaneous KS.

3. b. 4. i) patient enrollment requirements and clinical definitions of therapy response

The study was conducted at a single study centre and was designed to establish the activity and toxicity of thalidomide in the treatment of cutaneous AIDS-associated KS. Eligibility for enrollment was restricted to patients with serological evidence of infection with HIV-1, aged between 18 and 65, who had a histopathological diagnosis of Kaposi's sarcoma. The state of their KS had to be objectively assessable by the AIDS Clinical Trials Group (ACTG) criteria (Krown *et al.*, 1989). An European Clinical Oncology Group performance status of 3 was necessary and because of the teratogenicity of thalidomide only male patients were eligible. Informed consent was obtained and the study was conducted by approval of the Camden and Islington Community Health Services NHS Trust Research Ethics Committee.

The patient exclusion criteria were: active opportunistic infection, liver function tests >3 times normal, history of neuropathy or relevant drug hypersensitivity, systemic anti-KS therapy in the preceding month, previous local therapy to assessable lesions, Hb<10g / dl, platelets $<100 \times 10^9$ / l and neutrophils $<750 \times 10^6$ / l. Anti-retroviral and protease inhibitor therapy was noted throughout the study. Response evaluation required 8 weeks treatment

and was performed fortnightly by a single physician using ACTG guidelines. Toxicity to thalidomide was documented according to World Health Organisation toxicity grading (World Health Organisation, 1979).

The treatment schedule was 100mg thalidomide given orally at night for two months, thereafter continued therapy was offered. Blood samples were taken for analysis of HHV8 DNA at the start of thalidomide treatment and also 4 - 6 weeks into the 8 week study. HHV8 DNA titre in blood was determined by end-point titration of the purified blood DNA so that virological response to treatment could be determined. A 3 $\log_{10}$ change in titre was defined as being virologically significant. Only if both samples were taken was the patient described as virologically evaluable. All blood samples were stored and extracted using the Qiagen protocol described in section 2. c. 1.

Seventeen patients, median age 40 years (range 30 - 48) were enrolled. Their median CD4 count was 36 (range 4-552) and 9 of the 17 had prior AIDS-defining opportunistic infections. Sixteen of the 17 had received prior local therapy for KS and one had received oral 13-*cis* retinoic acid. Concomitant anti-retroviral therapy was being taken by 12 patients and was altered in 5 during the course of the trial. This was felt to be a confounding factor in only one patient who was commenced on the protease inhibitor indinavir two weeks after starting thalidomide.

3. b. 4. ii) clinical and virological response to therapy

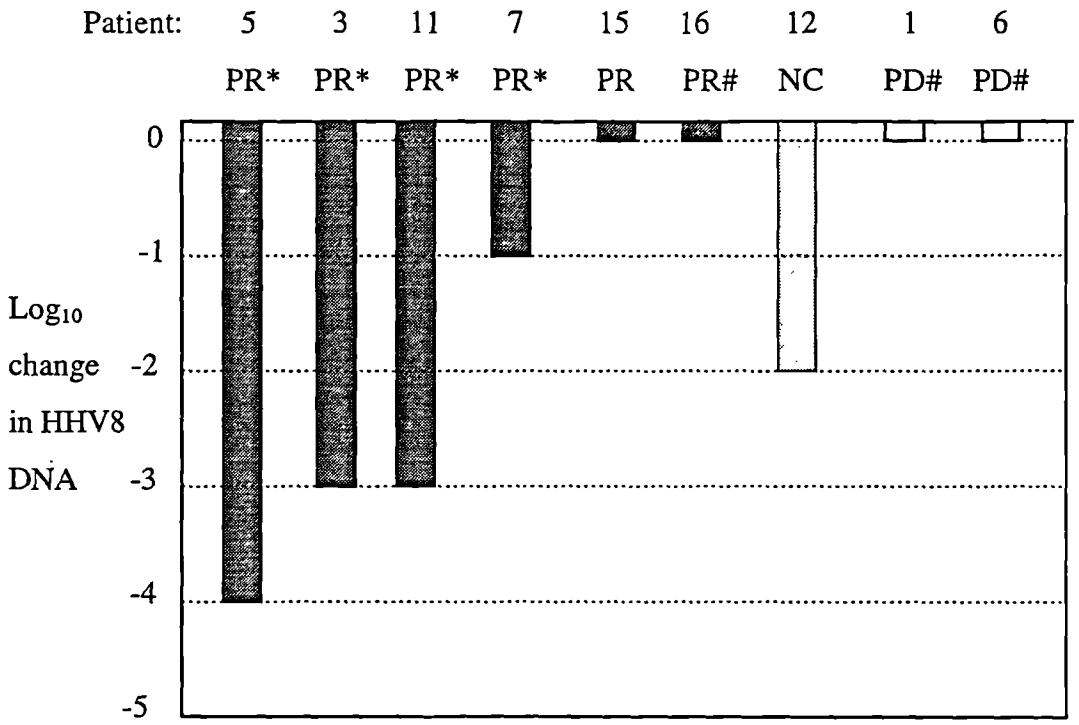
Nine of the 17 patients completed 8 week's treatment and were clinically evaluable for response. The 8 patients who were unevaluable, due to non-completion, withdrew between 1 and 4 weeks after starting thalidomide. Six patients had self-limiting toxicity: four developed a rash, one Raynaud's syndrome and one nausea. No rechallenge was undertaken and the majority of side-effect symptoms cleared quickly. One other patient was withdrawn owing to progressive disease and one for poor compliance. Of the 9 clinically

evaluable patients, who completed 8 weeks oral therapy with thalidomide, 6 patients achieved a partial clinical response, 1 patient had stable disease and two progressive disease. Duration of response was from 6 to 30 weeks after cessation of treatment.

Blood samples were available from all patients at the start of therapy at which point HHV8 DNA was detected in 10 / 17 (59%) patients.. Five of the 6 clinical partial responders were virologically evaluable (fig 3. 5. 1.); 3 patients (patients 3, 5 and 11) had a significant decrease in HHV8 DNA blood titre, with all 3 having undetectable HHV8 DNA after 4-6 weeks therapy. One partial responder (patient 15) had an unchanged titre and another (patient 7) a decrease in HHV8 DNA titre of $1\log_{10}$ which resulted in his HHV8 DNA becoming undetectable. The sixth patient with a partial clinical response (patient 16) was virologically unevaluable as HHV8 DNA was not detected in his blood at either the start of treatment, or after 4 - 6 weeks of treatment.

The one patient (patient 12) with no clinical response had a $2\log_{10}$ decrease in HHV8 DNA titre, although HHV8 DNA was still detectable in blood after 6 weeks of therapy. Both patients (patients 1 and 6) with progressive disease, who completed the 8 weeks treatment regimen, could not be assessed virologically as neither of them had detectable HHV8 DNA in their blood at the start of therapy or after 4 -6 weeks of therapy. It was however interesting to note that the one patient who was withdrawn because of progressive disease after 4 weeks of thalidomide (see above), but who did have the second blood sample taken at the 4 week time-point had a significant rise in viral DNA titre of 3 $\log_{10}$ (data not shown on figure). The patient who received indinovir therapy 2 weeks after starting thalidomide had stable disease, but was virologically unevaluable as HHV8 DNA was not detected in his blood at the start, or after 5 weeks, of thalidomide treatment.

Figure 3. 5. 1. Change in HHV8 DNA titre between baseline and after 4 - 6 weeks therapy with thalidomide in the 9 patients completing treatment course



PR: partial KS response

NC: no clinical change

PD: progressive KS

* patients who cleared systemic HHV8 DNA after 4 - 6 weeks treatment

patients with undetectable HHV8 DNA before and after 4 - 6 weeks treatment

3. b. 4. iii). study outcomes

The clinical response rate of patients receiving the full 8 weeks thalidomide was 66%, this figure dropping to 60% if the one patient who was unevaluable, due to disease progression, is included. Such a response rate compares favorably to with most other systemic chemotherapy's. Conventional chemotherapy response rates range from 25% for doxorubicin / bleomycin / vincristine (Gill *et al.*, 1996) to 75% for liposomal doxorubicin (Harrison *et al.*, 1995). HHV8 DNA was detected in approximately 60% of patients at the start of therapy an incidence similar to that previously shown in other sections of this thesis. Most significantly HHV8 DNA in blood was cleared in 4 of the 5 patients who completed the treatment regime, were virologically evaluable and had a clinical partial response, this clearance was, in 3 of the 5 cases, a drop of 3-4 $\log_{10}$ titre of viral genome. Such a correlation in clinical response to treatment and reduction in HHV8 DNA titre again strengthens the hypothesis that HHV8 DNA titre could be used as a surrogate marker for response early in the systemic treatment of KS. It should also be noted that in the one patient with progressive disease, who had to withdraw from the study, the HHV8 DNA titre rose by 3 $\log_{10}$ in 4 weeks, showing that both a decrease and increase in clinical disease is mirrored by HHV8 genome titre change.

In comparison to other therapies used for the treatment for early KS in HIV-infected individuals thalidomide would appear to have a number of positive advantages. The response rate in this study is higher than that found for other non-cytotoxic therapies such as α-interferon, which are only effective in patients with high CD4 counts. Also thalidomide is orally administered and has no associated haematotoxicity as do the systemic chemotherapy's. A number of patients did experience side-effects, such as rash, however these were mild and reversible on cessation of treatment. A similar study of oral thalidomide for cutaneous KS (Welles *et al.*, 1997) has suggested that by careful management of oral dose the mild side-effects experienced can be made clinically manageable while maintaining the effectiveness of the therapy.

3. c. Anti-HHV8 antibody prevalence in patients with haematological malignancies

3. c. 1. Introduction

Human herpesvirus 8 has been associated with a variety of malignancies including KS, BCBL and MCD (Chang *et al. 1994.*,Soulier*et al.*, 1995 and Cesarman *et al.*, 1995). Outside certain patient populations, notably HIV-infected gay men, these diseases are extremely rare. The prevalence of HHV8 infection in the UK general population has been shown to be between 1 and 5%, as defined by serological and genome analysis techniques, (Whitby *et al.*, 1995 and Simpson *et al.*, 1996). However, in 1997, Rettig and colleagues proposed an association between HHV8 and multiple myeloma (MM, Reittig *et al.*, 1997) a disease with no known predilection for particular sub-groups of the population.

Multiple myeloma is the second most frequent blood malignancy in north America, affecting approximately 40,000 individuals at any one time, with 13,000 new diagnoses made each year. The disease is characterised by the accumulation of immortalised plasma cells in the bone marrow, together with the high level production of monoclonal antibody from these clonal cells. This antibody is easily detected in both serum and urine. The systemic effects of this cellular imbalance include skeletal degeneration, due to the formation of osteolytic lesions, renal complications, due to the circulating levels of monoclonal antibody and an increased likelihood of opportunistic bacterial infections (reviewed in Vescio *et al.*, 1995). The median survival period for a patient diagnosed with MM is no more than 30 months, however bone marrow transplantation has been shown to be curative in 50% of patients who survive treatment (Gahrton *et al.*, 1991).

Monoclonal gammopathy of undetermined significance (MGUS) is the precursor condition to MM and is also characterised by the presence of monoclonal antibody in serum and urine, and an increased number of plasma cells in the bone marrow. However patients with MGUS do not suffer from the overt clinical symptoms associated with MM. Patients with MGUS have a 25% probability of progressing to full MM, regardless of therapeutic intervention (Kyle *et al.*, 1993).

Rettig and colleagues have reported that HHV8 DNA sequences could be detected in the bone marrow stromal cells of all 15 patients they investigated with MM, and that HHV8 proto-oncogene RNA transcripts, including the v-Il 6, could also been found in these tissues. The v-Il 6 homologue has previously been shown to have a similar *in vitro* activity to cellular Il 6 (c-Il 6) and is able to support the growth of c-Il 6-dependant cell lines in the absence of c-Il 6 (Moore *et al.*, 1996). Importantly c-Il 6 is a well defined growth factor for myeloma and may both stimulate myeloma cell growth and prevent the apoptosis of malignant plasma cells (Hirano *et al.*, 1991). It was suggested that the production and secretion of HHV8 v-Il 6 from infected stromal cells may drive the proliferation of plasma cell clones thus promoting the conversion of the pre-cancerous state of MGUS to full MM. Interestingly, this group found that 25% of MGUS patients also had detectable HHV8 DNA in their stromal cells, a similar percentage to that defined at risk of progression from MGUS to MM.

3. c. 2. Study of anti-HHV8 antibodies and viral genome prevalence in patients undergoing allogeneic bone marrow transplantation

3. c. 2. i). aims of the study

To investigate the possible association between HHV8 and haematological malignancies, particularly MM and MGUS, a cohort of patients undergoing allogeneic bone marrow transplantation (BMT) to correct haematological disorders were analysed. The presence of HHV8 DNA sequences in circulating leucocytes and serological evidence of previous infection with HHV8 was sought and correlated with the type of haematological malignancy the patient was being treated for.

3. c. 2. ii) patient profile and samples available for analysis

Serum and whole venous blood was available from 49 patients undergoing sibling-related and unrelated allogeneic BMT. Reasons for transplantation were: acute lymphocytic leukemia (n=13), non-Hodgkin's lymphoma (n=9), acute myeloid leukemia (n=7), multiple myeloma (n=6), Hodgkin's disease (n=5), chronic myeloid leukemia (n=4), chronic leukocytic leukemia, Burkitt's lymphoma, chronic granulocytic leukemia, myelo-dysplastic syndrome and unknown (all n=1). The serum came from samples received in the Department of Virology, UCLMS for routine pre-transplant screening for HIV-1, hepatitis B and C viruses and CMV. Pre-transplant sera were used to avoid confusion with any antibodies subsequently produced by the B-lymphocytes of the transfused bone marrow. Ethical permission for testing was received by Chairman's action from the Middlesex Hospital Ethics Committee.

The presence of antibodies for HHV8 was determined by two previously published methods; an indirect immunofluorescence assay (IFA) using the HHV8-containing cell-line BCP-1, detecting antibodies directed against the HHV8 latent nuclear antigen (LAN) and by an enzyme-linked immunoabsorbant assay (ELISA) system utilising the carboxy-terminal section of the HHV8 orf 65 gene product as an antigen target (Simpson *et al.*, 1996). Sera were prepared and the assays performed as described in sections 2. d. and 2. e.

Venous blood samples were received for routine post-transplant surveillance monitoring of systemic CMV DNA (see section 3.d.). CMV is a pathogen associated with severe patient morbidity and mortality in these acutely immunosuppressed patients (Meyers *et al.*, 1984). For screening of HHV8 genome presence, the first blood sample received post-transplant was prepared using either the glycigel extraction protocol or the QIAGEN commercial extraction protocol, (see section 2. c. 1). Regardless of the extraction methodology the DNA equivalent to 10µl of blood was added to the first round of the HHV8 nested PCR.

If there was serological evidence of previous HHV8 infection in the pre-transplant serum sample, by either serological assay, or HHV8 DNA was detected in the first post-transplant surveillance blood sample then all available blood samples from that patient were also analysed by the HHV8 PCR. Following the analysis for antibodies and viral DNA described above the identity of the patients was made available. After unblinding of the patient details those with a diagnosis of MM had DNA extracted, as before, from all available blood samples and the presence or absence of HHV8 genome sequences in each sample determined.

3. c. 2. iii) study outcomes

Of the 49 patients investigated 4 had pre-transplant evidence of previous antibody production to HHV8 infection. Of these 4, none had a diagnosis of MM. Two patients had antibodies detected by both the IFA and ELISA, one patients had antibodies detected by the IFA only and one by the ELISA only (Table 3. 6. 1.). One of the patients who was HHV8 antibody-positive also had detectable HHV8 DNA in their one available post-transplant blood sample. This sample had been taken 6 weeks after transplantation. None of the other three patients with anti-HHV8 serum antibodies had detectable HHV8 DNA in their blood samples at any time post-transplant. Blood samples taken up to four months post-transplant were available from each of these four patients.

Of the 45 patients who did not have detectable HHV8 antibodies by either serological assay none had detectable HHV8 DNA in their first post-transplant blood sample. The six patients with MM were all part of this group of 45 patients with no serological evidence of a previous HHV8 infection. Three of the six with MM had a single blood sample taken post-transplant, one had blood samples available until one month post-transplant, the other two patients each had blood samples available up to six months post-transplant. None of the blood samples taken from patients with MM contained detectable HHV8 DNA.

3. c. 3. Discussion

The overall prevalence of anti-HHV8 antibodies in this group of 49 patients receiving BMT for a variety of haematological disorders was, at 8%, little different from the declared rate of between 2 and 5% in the UK general population (Simpson *et al.,* 1996). There was no serological evidence to support the recent suggestion of HHV8 involvement in MM and / or MGUS,

Table 3. 6. 1. Detection of HHV8 DNA and anti-HHV8 antibodies in 49 patients undergoing allogeneic BMT, (original haematological malignancy shown in brackets).

	HHV8 IgG detection pre-transplant		**HHV8 DNA in post-transplant blood samples**
	IFA	ELISA	
Patient 1 (lymphocytic leukemia)	**+ve**	**+ve**	not detected
Patient 2 (lymphocytic leukemia)	**+ve**	-ve	not detected
Patient 3 (non-Hodgkin's lymphoma)	-ve	**+ve**	detected in single available sample
Patient 4 (Hodgkin's disease)	**+ve**	**+ve**	not detected
Patients 5 - 10 (all with multiple myeloma)	-ve	-ve	not detected
Patients 11 - 49 (various haematological diseases, see text)	-ve	-ve	not detected

indeed none of the patients with these disorders were found to have been infected with HHV8 by serological analysis. The only patient who had detectable HHV8 DNA in peripheral blood, who also had circulating antibodies to the virus, was born in an area in which classical, HIV-unassociated KS is relatively common. In such areas the prevalence of HHV8 infection in the general population may be as high as 35% (Gao *et al.*,1996 and Miller *et al.*, 1996). The other 3 patients with antibodies to HHV8 had a variety of unrelated haematological malignancies. These three patients with serological, but not genome detection, evidence of previous HHV8 infection were, as were all the patients in the study, in a period of acute immunosuppression. It was interesting to note that even with such severe damage to their immune control HHV8 DNA could not be detected in the systemic circulation suggesting that no reactivation of the virus from latency had occurred. It is possible that the extreme levels of leucopenia experienced by these patients may, however, have reduced the levels of latent HHV8 present in their blood.

The low rate of HHV8 seropositivity in this study group would appear to preclude any direct etiological association between HHV8 and the haematological diseases found in these patients, including MM and MUGS. While the molecular mechanisms proposed by Rettig and colleagues for the interaction of HHV8 v-IL 6 with plasma and dendritic cells in patients with MM would appear to have theoretical merit the absence of current or previous HHV8 infection in any of the myeloma patients in this study seems to suggest that such an association is extremely unlikely.

Recently, the findings of this study have been confirmed by a variety of international investigations, which also found no association between the presence of HHV8 DNA in peripheral blood, aspirated bone marrow or bone marrow stromal cells and MGUS / MM (Parravicini *et al.*, 1997, Masood *et al.*, 1997 and Whitby *et al.*, 1997). Neither was an association found between individuals with circulating anti-HHV8 antibodies and these two conditions. At present Rettig and colleagues remain convinced of their initial findings and

have been supported by one other study which detected HHV8 DNA in the bone marrow biopsies of 18 of 20 patients with MM (Rettig *et al.*, 1997a and Brousset *et al.*, 1997). However the inability of the other groups, as well as ourselves, to provide supporting data especially of a serological nature, must cast grave doubt on this proposed virus / disease association.

3. d. Establishment of a CMV DNA surveillance service for patients receiving allogeneic bone marrow transplantation

3. d. 1. Introduction

Bone marrow transplantation is an increasingly common method of treatment for a variety of haematological malignancy, aplastic anaemia and genetic diseases (reviewed in O'Reilly, 1983 and Hiemenz *et al.*, 1993). Although the rates of complications in bone marrow transplantation, such as graft versus host disease (GVHD), have decreased in recent years the immunosuppressive regimes necessary to facilitate donor marrow engraftment still place patients at significant risk of developing a range of opportunistic viral infections (Brayman *et al.*, 1992 and Winston *et al.*, 1990). Potentially the most severe of these are associated with CMV infection (Meyers, 1984 and Weiner *et al.*, 1986).

Before transplantation it is essential to determine, by serology, whether a bone marrow recipient has previously been infected with CMV. If the patient has no evidence of CMV then the importance of using donor marrow originating from a similarly CMV-seronegative donor is increased. Providing such a "negative-to-negative" donation is possible the recipient is at extremely low risk of post-transplant CMV disease. Indeed such disease would only occur if the recipient was exposed to CMV from an external source post-transplant, such as a contact actively excreting CMV, or a CMV-containing blood component. Even more importantly, if the recipient is found to be CMV-seropositive then it is imperative that the donor marrow also originates from an individual who is CMV-seropositive. If bone marrow from a CMV seronegative donor is transplanted into a CMV seropositive recipient then the introduced bone marrow, which will supply the recipient with all the immunological cells necessary for establishment of an immune system, will have had no previous experience of CMV. The recipient is hence at great risk of reactiviation of their own latent CMV. This may lead to a severe CMV infection because this

will essentially be a primary infection to the new immune system. Such "negative-to-positive" transplants have the highest associated risk of CMV disease, with a number of studies showing that over 70% of such patients develop CMV infection (reviewed by Winston, *et al.*, 1990). An intermediate risk of CMV disease is associated with CMV seropositive donation to a CMV seropositive recipient and CMV seropositive donation to a CMV seronegative recipient.

CMV disease usually appears between 3 and 16 weeks after transplantation (Meyers *et al.*, 1980). Persistent, unexplained, fever and non-specific symptoms in the presence of cultures negative for bacteria and fungi are frequently the initial presenting symptoms. Such patients may also develop other symptoms, such as hepatitis, leucopenia or thrombocytopenia, these are often difficult to distinguish from GVHD (Winston, 1993). Unfortunately the existing diagnostic procedures for CMV; viral culture and the DEAFF test, are often insensitive and have been found to be influenced by the cytotoxic effects of the blood and urine samples analysed (Fedorko *et al.*, 1989 and Stephan *et al.*, 1997). Because of this a number of centers have switched to the use of DNA assays, such as PCR, to monitor CMV genome load in patients at risk of developing CMV-associated post-transplant disease (Sandin *et al.*, 1991, Vleiger *et al*, 1992 and Storch *et al.*, 1994).

When CMV infection, be it primary or from reactivation, occurs in the BMT patient, prior to functional establishment of the donor marrow, the greatest clinical risk is the development of CMV pneumonitis. Such a pneumonitis may have a rapid onset with fulminant respiratory failure developing over 2 - 3 days in association with diffuse interstitial infiltrates (Winston *et al.*, 1990). Studies have found that up to 15% of allogeneic BMT recipients will develop CMV pneumonitis in the absence of effective chemoprophylaxis (Wingard *et al.*, 1988). CMV infection of the lung is the most frequent cause of pneumonia in this patient population and is associated with a high level of patient morbidity and mortality. Untreated over 90% of all patients with a CMV pneumonitis

will die in the first 3 weeks after presentation of clinical symptoms (Prentice *et al.*, 1994).

Originally it was presumed that CMV pneumonitis was due to direct viral lysis of pulmonary cells. However it has been shown subsequently that CMV pneumonitis is rare in autologous transplants where the patient's own bone marrow is re-engrafted, but is far more common in allograft recipients (Appelbaum *et al.*, 1982). This pattern is mirrored by the incidence of GVHD in these patients and suggests that the immunological events associated with GVHD may be involved in the development of CMV pneumonitis. It is now known that both GVHD and CMV pneumonitis are associated with an increase in level and activity of cytotoxic T-cells in the lung (Bowden *et al.*, 1987 and Grundy *et al.*, 1987). When combined with data from nude mice studies, showing that CMV replication in the lung in the absence of T cells does not lead to the development of pneumonitis, it is now thought that CMV pneumonitis is caused by the inappropriate response of a T-cell subset to the viral infection rather than by direct virucidal lysis of infected cells (Shanley, 1987).

Even after rapid therapeutic intervention the mortality rate in patients with CMV pneumonitis can still be as high as 40% (Reed *et al.*, 1988). Because of the significance of CMV-associated pulmonary disease a number of prophylactic treatments have been examined in those patients receiving intermediate, or high risk BMT's. These usually involve long-term use of one of the anti-herpesvirus chemotherapeutic agents such as acyclovir (ACV), foscarnet (PFA) or ganciclovir (GCV), either alone, or in combination with CMV hyper-immune IgG preparations (Prentice *et al.*, 1994, Bowden and Meyers, 1990 and Winston *et al.*, 1990).

Whilst the use of prophylactic therapies has been associated with the efficient prevention of CMV disease post-transplant the two most effective anti-CMV chemotheraputics, PFA and GCV, are both highly toxic. Of particular concern is treatment with GCV, a bone marrow suppressive cytotoxic drug, which may

result in severe neutropenia and a delay in bone marrow engraftment (Schmidt *et al.*, 1991). Prolonged neutropenia increases the risk of developing a range of other opportunistic infections, particularly bacterial septicaemia and fungal pneumonia. Studies have found that even though GCV is effective in preventing CMV disease post-transplant, because of the associated neutropenia there is no overall improvement in rates of patient mortality when use of GCV against placebo is compared up to 6 months after transplantation (Goodrich *et al.*, 1993). Foscarnet also has significant *in vitro* activity against CMV, but is a potent nephrotoxic drug (Jacobson *et al.*, 1988). As a result of these toxicities a number of studies have considered the use of ACV (Prentice *et al.*, 1994 and Meyers *et al.*, 1988). ACV is usually indicated for the treatment of α-herpesvirus infections such as HSV-1, HSV-2 and VZV in immunocompromised individuals. Although ACV has a lower anti-CMV activity than GCV and PFA (because of the lack of production of a thymidine kinase by CMV) it has now been found to be relatively effective, when compared with placebo, in preventing emergence of CMV from latency in post-transplant BMT patients. One study found that 22% of patients on ACV prophylaxis developed CMV disease post-transplant whereas 38% of patients receiving no anti-herpesvirus prophylactic treatment developed disease (Bowden and Meyers, 1990)*. In this study there was no noticeable ACV-related toxicity in the treated group.

* This was a statistically significant protective effect (Chi squared test: P = 0.0025)

Important in the management of patients post-transplant is the effective diagnosis of active CMV infection. Ideally such a diagnosis must be made prior to the development of clinical symptoms, a point at which the effectiveness of treatment is reduced. Until recently the two most commonly used methods of diagnosing CMV infection have been conventional tissue culture and rapid-viral culture techniques such as the DEAFF test (Gleaves *et al.*, 1985). Conventional culture is an inappropriate method for the diagnosis of CMV in this setting as results may take up to 3 weeks to become available and low-level CMV viraemia may still be present even when viral culture has proved negative (Reed *et al.*, 1988). The introduction of the DEAFF test has

resulted in a much more rapid diagnostic analysis, usually within 24 hours. However this technique is relatively insensitive when compared with culture and has associated technical problems, such as a percentage of unreadable results due to toxicity of the sample for the cell monolayers used in the analysis. Molecular techniques, including PCR, have been investigated as potential alternatives for the detection of CMV. Preliminary results have shown that PCR analysis for CMV DNA in the venous blood of post-transplant BMT patients is a highly sensitive technique when compared to existing culture methodologies (Stephan *et al.*, 1997, Vlieger *et al.*, 1992 and Sandin *et al.*, 1991). Detection of CMV DNA in blood may also act as an early predictor of disease compared to culture (Storch *et al.*, 1994). Comparison has been made between the detection of CMV DNA in whole blood and detection in cell-free plasma (Woo *et al.*, 1997 and Laue *et al.*, 1997). It is theoretically possible that detection of CMV DNA in whole blood may be due to the presence of low-titre latent viral genome which is clinically unimportant. This may lead to inappropriate treatment of patients who are at no increased risk of disease. The detection of CMV DNA in plasma is likely to reflect the active production of mature virions and have a higher predictive value with regards to the development of CMV-associated disease.

3. d. 2. Development of improved clinical and virological protocols for the management of post-transplant BMT patients at UCL Hospitals

Until 1996 patients receiving allogeneic BMT at UCL Hospitals were monitored for CMV infection post-transplant by weekly, or twice monthly, culture and DEAFF analysis of whole blood and urine. All patients who were either pre-transplant CMV sero-positive, or whom received bone marrow from a CMV sero-positive donor received prophylactic GCV for 6 months post-transplant.

Following an audit in 1996 of the CMV surveillance service provided by the Department of Virology, UCLMS it was decided to evaluate alternative methods of CMV detection in patients at risk of developing CMV disease. The 1996 audit raised several areas of potential concern in the service being provided, especially in the use of DEAFF analysis as a surveillance technique for early detection of CMV infection. As can be seen, in table 3. 7. 1., 41% of blood samples analysed by the DEAFF test were toxic to the cell monolayer used in the assay which rendered the result void, a slightly lower incidence of test failures were seen in the analysis of urine and throat swab samples. Combined with the 3 week period necessary to obtain a definitive negative viral culture result this prompted the investigation of PCR for detection of CMV DNA in whole blood and cell-free plasma as an alternative methodology for identifying patients with clinically significant, active, CMV infection.

Another factor influencing a change in the surveillance service was an alteration in the post-transplant management of the BMT patients. The levels of neutropenia associated with the use of prophylactic GCV had raised concern as to the overall patient morbidity and mortality associated with such wide-spread use of this drug, even though this protocol had protected all patients treated from overt CMV disease. It was decided to change from the use of prophylactic GCV to prophylactic ACV. Although ACV has less associated toxicity than GCV it has been well documented that ACV does not provide such an effective protection against the development for CMV disease (Bowden and Meyers, 1990). By incorporating an aggressive strategy of PCR detection of CMV in blood, and rapid intervention with high dose intravenous GCV in those patients with detectable CMV DNA it was hoped that CMV disease could be prevented, while minimising the overall levels of therapy toxicity.

Table 3. 7. 1. Department of Virology, UCLMS, 6 month audit of laboratory results for CMV surveillance by the DEAFF test in allogeneic BMT recipients

Sample type	No. of samples	CMV		
		detected (%)	**not detected** (%)	No. toxic (%)
Heparinised Blood	60	1* # (1)	35 (58)	24 (41)
Urine	62	4* (7)	43 (43)	15 (24)
Throat Swab	35	0	24 (69)	11 (31)

* CMV was grown in conventional culture from all these 5 samples

this patient received ACV, rather than GCV, prophylaxis

3. d. 2. i) patient samples taken for CMV surveillance analysis and frequency of sampling

To allow comparison with the 1996 audit the existing system of CMV surveillance, by conventional culture and DEAFF test, was continued. For this purpose 20ml heparinised peripheral blood samples were taken every week post-transplant for the first 12 weeks and then twice monthly thereafter until 26 weeks post-transplant. Protocols for CMV detection using these methods were as described by Gleaves and colleagues (Gleaves *et al.*, 1984). The DEAFF and culture analysis were performed by members of the Virus Isolation Section, The Virology Department, UCL Hospitals NHS Trust.

For analysis of CMV DNA 5 ml EDTA-preserved peripheral blood was taken at the same time-points as the heparinised peripheral blood. The EDTA blood was processed as described in section 2. c. 1. and the DNA prepared using the Qiagen DNA extraction protocol described in section 2. c. 1. After extraction CMV PCR analysis was performed on 10 µl of extracted DNA (equivalent to the DNA present in 10 µl of the whole blood sample). This analysis gave a limit of detection sensitivity for CMV DNA of 100 copies / ml of blood as the CMV PCR has previously been shown to have a detection sensitivity of a single copy of CMV DNA (Mitchell *et al.*, 1994). The plasma was prepared for PCR analysis as described in section 2. c. 2., DNA prepared from 5µl of plasma was analysed for the presence of cell-free CMV DNA.

3. d. 2. ii) chemotherapeutic intervention with anti-CMV therapy post-transplant

All patients received prophylactic ACV throughout their first 26 weeks post-transplant. However if a patient presented with clinical symptoms suggestive of CMV disease a two week period of intravenous GCV was started and

continued until symptoms resolved. If a patient with no obvious symptoms had detectable CMV DNA in peripheral blood then a repeat sample was taken 1 week later, if CMV DNA was still present in the blood (a "consecutive +ve") then induction GCV therapy was also initiated and continued until CMV DNA could no longer be detected in the blood. However if the blood sample taken a week later did not contain CMV DNA, and there were no other markers of clinical or virological CMV, normal PCR surveillance was continued without therapy. Following initiation of GCV therapy blood samples were then taken weekly for investigation of CMV DNA response. Until CMV DNA became undetectable anti-CMV therapy was continued. Throughout the study GCV was the first treatment of choice, however because of toxicity, adverse drug reaction and / or a failure to clear CMV a number of patients received PFA treatment as an alternative to GCV.

3. d. 2. iii) patient profile and period of study

The above protocol for management of post-allogeneic BMT CMV infection was initiated in October 1996 and continued until May 1997. Only patients who provided a full set of surveillance samples, commencing immediately after transplant and continuing until at least day 60 post-transplant, were included in the analysis.

The total number of patients undergoing BMT during this period was 32 (14 female, 18 male). The median age of these patients was 32 years (range 16 - 46). In 14 transplants both donor and recipient were CMV-seropositive, in 13 both were CMV-seronegative and in 5 transplants the donor was CMV-seronegative and the recipient CMV-seropositive. All patients who were CMV-seronegative received blood and blood products post-transplant which were known to come from CMV-seronegative donors.

3. d. 2. iv) clinical outcome in the 32 patients receiving a BMT during the study period

Of 32 patients who received a BMT during the study period 29 survived beyond day 20 post-transplant to at least day 60. Of these patients the mean follow-up time was 124 days. None of the patients in the study had any clinical evidence of CMV during the period of the study. Two cases of fatal pneumonitis did occur, however cause was not established due to refusal for post-mortem examination. Neither of the 2 patients had CMV DNA detected in any sample taken during the post-transplant period or clinical symptoms suggestive of CMV involvement.

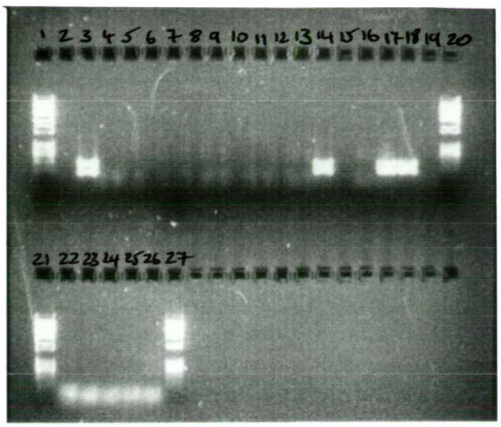
3. d. 2. v) detection of CMV infection by conventional culture and DEAFF

Only 2 patients had evidence of CMV infection by conventional CMV culture (table 3. 7. 2.). Each patient had a single culture-positive result. At these times both patients had concurrent CMV DNA in their blood when analysed by PCR. The DEAFF test was never positive for any patient. Sample toxicity in the DEAFF and culture tests remained a significant problem; overall 23% of DEAFF tests were invalid because of the toxic effects of the samples on the HEL monolayer used in the assay, 6% of culture tests were invalid for the same reason. Half the patients in the study had at least one incidence of sample toxicity in the DEAFF and culture analysis.

3. d. 2. vi) evaluation of CMV infection by detection of CMV DNA in blood

The average number of blood samples available for analysis from each patient was 15. Of the 456 blood samples analysed, 93 (20%) contained detectable CMV DNA (table 3. 7. 2.). Fourteen of the 29 patients had at least one sample with detectable CMV DNA. Thirteen of these 14 had consecutive +ves leading to initiation of GCV therapy. In these 13 patients there were a total of 22

Figure 3.7.1.1. Detection of CMV DNA in blood from patients receiving a bone marrow transplant



Lanes 1, 20, 21 and 27: molecular weight marker - ϕX 174 cut with *Hae III* band sizes in nucleotides (from bottom up) 72, 118, 194, 234, 271, 310

Lanes 3 and 14: samples containing detectable CMV DNA

Lanes 2, 4 - 13, 15 and 16: samples containing no detectable CMV DNA

Lanes 17: CMV DNA +ve control containing c. 10 copies CMV DNA

Lane 18: CMV DNA +ve control containing c. 1 copy of CMV DNA

Lane 19: CMV DNA +ve control containing < 1 copy CMV DNA

Lanes 22 and 23: negative controls containing water added with reagent pipettes

Lanes 24 - 26: negative controls containing water added with sample pipettes

Table 3. 7. 2. Detection of post-transplant CMV infection by culture, DEAFF and CMV PCR in 29 patients receiving a BMT and surviving to day-60 post-transplant

CMV detection protocol	Samples			Patients		
	Tested	Toxic	**Positive**	**Tested**	With one or more toxic samples	With one or more positive samples
CMV culture	345	19 (6%)	**2**	29	18	**2**
DEAFF test	346	81 (23%)	**0**	29	24	**0**

CMV PCR	**Tested**	**CMV DNA detected**	**Tested**	**CMV DNA detected at least once**
whole blood	456	**93** (20%)	29	**14**[#]
plasma	263	**23*** (9%)	29	**10**

* all 23 plasma samples with detectable CMV DNA came from the whole blood samples found to contain detectable CMV DNA.

13 of these 14 patients had consecutive CMV DNA +ves in whole blood which initiated the use of anti-CMV therapy

consecutive +ve events, all of which were treated. Seven patients had one episode, 4 had 2 episode, 1 had 3 and 1 had 4 episodes.

Plasma samples were available from all 93 whole blood samples which tested positive for CMV DNA, 23 (25%) of these plasma samples contained CMV DNA. A total of 170 plasma samples from blood which did not contain detectable CMV DNA were also analysed, none of these had detectable CMV DNA. In 6 of the 22 episodes of blood CMV DNA consecutive +ve's CMV DNA remained completely undetectable in the corresponding plasma samples.

3. d. 2. vii) donor / recipient status of patients having at least one blood sample with detectable CMV DNA.

Twelve of the 14 CMV-seropositive recipients of CMV-seropositive donor marrow had at least one episode of detectable CMV DNA in blood, these 12 including both patients with positive culture results. Two of the five CMV-seropositive recipients of CMV-seronegative donor marrow also had detectable CMV DNA in blood at some time during the study. None of the 13 CMV-seronegative recipients of seronegative marrow (all of whom received CMV-ve blood, blood products and marrow) had CMV DNA detected in their blood at any time post-transplant.

3. d. 2. viii) response of systemic CMV DNA titre to GCV and PFA therapy

Figure 3. 7. 1. shows the post-transplant timing of anti-CMV therapy in the 13 patients in which it was initiated. Every patient who had an episode of consecutive +ve CMV DNA in blood had their first CMV DNA+ve result in the first 60 days post-transplant. All 22 incidences of consecutive +ves were treated with anti-CMV therapy and all patients except one (who was treated twice with PFA) were initially treated with GCV. In 6 of the 20 GCV treatment episodes the initial 2 week GCV therapy was insufficient to clear

Figure 3. 7. 1. Time points of anti-CMV therapy initiation in 13 patients with at least one consecutive +ve episode of CMV DNA detected in peripheral blood

Days post-transplant	1st treatment (13 patients)	2nd treatment (6 patients)	3rd treatment (2 patients)
0 - 10			
11 - 20			
21 - 30	6		
31 - 40	4		
41 - 50	2		
51 - 60	1	2	
61 - 70		1	
71 - 80			
81 - 90		2	
91 - 100			
101 - 110		1	
111 - 120			1*
+ 120			1

* this patient required a 4th treatment course, started on day 189.

detectable CMV DNA from the blood, however when therapy was switched to PFA all samples eventually became CMV DNA -ve. Clearance was in 1 week (3 patients) to 2 weeks (3 patients) after this therapy switch. The other patient who had two PFA treatments, cleared CMV DNA from their blood within 2 weeks of the initiation of each PFA therapy course.

3. d. 3. Discussion

The significance of CMV disease in patients receiving allogeneic bone marrow transplantation has been well described. The prophylactic use of GCV in this patient group has been found to have a protective effect against the development of CMV disease but overall patient survival rates are not increased due to the increased neutropenia experienced as a consequence of GCV use. The alternative use of ACV has been considered (Bowden and Meyers, 1990) and although not as active against CMV as GCV ACV has been found to have a protective effect against the development of CMV disease, without associated neutropenia and bone marrow toxicicty (Wolfe *et al.*, 1993). Recently a number of studies have shown that detection of CMV DNA in the blood and plasma of patients post-transplant is highly predictive of the development of active CMV disease and may be an effective marker for the instigation of aggressive anti-CMV therapy to prevent, or modulate, disease (Storch *et al.*, 1994).

In this study the use of ACV prophylaxis was examined in concert with a PCR surveillance protocol directing GCV or PFA therapy to patients with repeated episodes of detectable CMV DNA in their blood. Of 29 patients who survived the first 20 days post-transplant none subsequently developed CMV disease during the study. This included 5 CMV-seropositive patients who received CMV-seronegative donor marrow and hence were at the highest risk of CMV disease development. CMV DNA was detected at least once in 14 of 29 patients, with anti-CMV therapy required at least once in 13 patients. Although all treatment episodes resulted in the eradication of CMV DNA from

peripheral blood there were 6 cases in which a therapy switch from GCV to PFA was first required and further treatment in response to the re-emergence of CMV DNA in blood was necessary in 6 patients. The re-emergence of CMV DNA suggests that latent virus is an important reservoir for subsequent infection and that the immunological environment in these patient remained favourable for reactivation up to 6 months post-transplant (the latest episode of consecutive +ves was 180 days post-transplant, see fig. 3. 7. 1.)

None of the 13 patients who were CMV sero-negative before transplantation and who received bone marrow, blood and blood products from sero-negative donors had detectable CMV DNA in their blood at any time. This important observation suggests that such a rigorous surveillance protocol may be unnecessary for this type of patient, and that the screening of bone marrow donors and blood products for use in these patients remains an extremely effective precaution in preventing CMV infection.

CMV DNA was detected much more frequently in whole blood than in plasma. As CMV is a predominantly cell-associated virus this finding was unsurprising. Previously patients with CMV DNA in their plasma have been found to be much more likely to develop active CMV disease than patients with virus present only in whole blood (Woo *et al.*, 1997). The implication being that detection of the viral genome in a cell-free environment is indicative of an active systemic infection, whereas detection in whole blood may be due either to sub-clinical levels of virus or to the presence of latent virus. With the absence of CMV disease in this patient study group it is difficult to confirm or deny the significance of these findings. However it appears unlikely that the CMV DNA detected in whole blood originates from latent virus, as after every treatment episode the previously detectable CMV DNA was eradicated. Treatment with PFA or GCV would not have affected latent genome load in such a fashion as both therapies are only active against replicating virus through the inhibition of viral genome replication.

Examination of the time post-transplant at which systemic CMV DNA was first detected indicates that the first 60 days following transplant are the period of highest risk for development of a CMV infection. Indeed no patients had their first episode of detectable CMV DNA after this time. If this finding is repeated in ongoing studies it may be possible to modulate the surveillance protocol to reduce the amount of analysis required for each patient who reaches this time point without their first episode of detectable systemic CMV DNA.

When compared with established diagnostic assays, such as DEAFF and culture, PCR is vastly more sensitive in its ability to detect CMV DNA in whole blood. It is also free from the associated problem of sample toxicity, as all the blood DNA analysed is purified before analysis in the PCR assay. Whether such assay sensitivity is actually required cannot be answered by this study as no comparison was made with patients relying on conventional techniques alone, and none of the study patients developed CMV disease. However after the conclusion of the study it was decided to cease use of culture and DEAFF in the surveillance of these patients and to rely solely on PCR. At the time of writing there are still no BMT patients who have developed CMV disease while enrolled in this protocol.

The use of prophylactic ACV, coupled with PCR surveillance and GCV / PFA intervention, has been remarkably effective in preventing the life-threatening CMV disease common in this patient group. Data from previous studies would suggest that (apart from the 13 CMV-seronegative recipients of sero-negative morrow) these patients, without CMV prophylaxis, would experience a 15 - 20% mortality rate due to CMV infection (data combined from Meyers *et al.*, 1988, Weiner *et al.*, 1988 and Winston *et al.*, 1990).

Although the incidence of post-transplant neutropenia has been greatly reduced from 1996 levels, when GCV rather than ACV was the prophylactic treatment, there has been at least one neutropenia-associated complication (of a pneumonic PCP infection) during the study period. In future it is hoped that

alternative therapies to intravenous GCV, such as oral GCV and cidofavir may be used in place of intravenous GCV to combat this problem. The requirement to treat patients after only 2 consecutive positive episodes of CMV DNA may be further examined, so that treatment is more targeted at those patients with the highest risk of CMV disease. The use of quantitative PCR techniques for determining systemic CMV genome load may also allow those patients at greatest risk of developing disease to be identified and specifically treated (Ljungman *et al.*, 1996).

3. e. Herpesvirus infections of the eye and central nervous system in patients with late-stage AIDS

3. e. 1. Introduction

As described in previous chapters herpesviruses have been associated with a wide spectrum of diseases in immunosuppressed patient populations. In individuals with chronic immunosuppression due to infection with HIV-1 the most common sites of severe herpesviral-associated disease are the eye and central nervous system (CNS), (reviewed in Steiner, 1996 and Ugen *et al.*, 1992). CMV is, in particular, a significant pathogen in these patients if their risk factor for HIV-1 infection is homosexual contact. Such patients have been found to have an almost 100% seroprevalence for CMV, with up to 50% actively shedding CMV in saliva or urine (Buimovici-Klein *et al.*, 1988). Many HIV-infected gay men are infected with multiple strains of CMV (Leach *et al.*, 1994) and it would appear that sexual transmission of CMV in this population is a significant factor in these high exposure rates (Drew *et al.*, 1984). Overall homosexuality, in contrast to other routes of HIV acquisition, has been found to be significantly associated with the development of CMV disease after HIV seroconversion (Gallant *et al.*,1992).

Cytomegalovirus retinitis is the most common ocular disease in patients with AIDS and is characterised as a severe necrotising infection of the retina, which if untreated, will result in the irreversible loss of sight in the affected eye (Bloom and Palestine, 1988). In 1992, in the United States, 20,000 cases of CMV retinitis were diagnosed, with approximately 25% of HIV-infected gay men likely to develop this disease at some point during their AIDS-associated illness (Katlama, 1993).

Although CMV retinitis frequently presents unilaterally as a granular retinal opacification, with or without associated haemorrhage, it is common for the

other eye to also become infected with the potential for complete blindness. Diagnosis is made clinically by fundoscopic visualisation of the damaged retina, however several studies have shown the use of PCR analysis of ocular fluid to be an appropriate method for discriminating between CMV retinitis and a host of other intra-ocular infections such as ocular *toxoplasma gondii* and *Pneumocystis carinii* choroiditis (Mitchell *et al.*, 1994 and Fox *et al.*, 1991). Following a diagnosis of CMV retinitis treatment is usually with systemic GCV or PFA, depending on the patient's tolerance of these drugs (reviewed in Drew, 1992). Intra-ocular treatment with GCV has been attempted but is of limited use as it does not prevent the bilateral spread of the virus, or CMV infection of other organs as effectively as a systemic treatment (Heinemann, 1989). Treatment with either anti-CMV therapy will halt the spread of retinal destruction, but improvement of visual acuity is limited. Patients must remain on therapy indefinitely to prevent disease progression, however such long-term treatment is associated with the generation of drug-resistant viral strains and may result in significant nephro- or myelotoxicity (Henderly *et al.*, 1987, Jabs *et al.*, 1987, Jacobson, 1989 and Baldanti *et al.*, 1995).

Cytomegalovirus is also a common pathogen in the CNS of AIDS patients. As with CMV retinitis, CMV infection of the CNS usually occurs in patients who are extremely immunosuppressed, predominantly those with CD4 counts below 100. Studies have shown that the risk of developing CMV disease at an ocular, or neurological, site may be as high as 40% when a patient's CD4 count is below 50 (Pertel *et al.*, 1992) and up to 20% of patients with CMV retinitis will develop neurological CMV infection at some point during their ocular disease. Neurological infection with CMV has a predicted mortality of 50% within 3 months of diagnosis if anti-CMV therapy is not initiated (Peters *et al.*, 1991b). The most common presentation of CMV infection in the CNS is as an encephalitis, although polyradiculopathy and myelitis have also been described (Holland *et al.*, 1994 and Marmaduke *et al.*, 1991). In the CNS, CMV has been shown to infect a wide variety of cells including neurons, astroglial cells and endothelial cells (Myerson *et al.*, 1984).

Entry into the CNS is from the blood, via the blood / brain barrier with the virus further disseminated by the CSF before infection of the brain parenchyma (Gray *et al.,* 1988). As mentioned in section 1. a. 7. CMV and HIV-1 may co-infect similar cell types, including neurological cells, and in the CNS are likely to interact thereby upregulating the replication of both virus types (Belec *et al.,* 1990). Such up-regulation may increase the severity of neurological disease associated with joint HIV and CMV infection (Wiley and Nelson, 1988).

Conventional diagnostic methods such as culture of the CSF or blood are of little use in diagnosing CMV infection of the CNS, due to their low sensitivity for infection at this site (Katlama, 1993). Symptomology may also be confused because of the similarity with other HIV-associated neurological disease such as HIV dementia. Molecular detection of CMV genome in CSF, or brain biopsy, by PCR has therefore become the usual diagnostic method of choice (Holland *et al.,* 1994). The identification of CMV as the aetiological agent of CNS disease is vital if appropriate therapy is to be initiated for maximum patient benefit.

A number of other herpesvirus ocular and CNS infections have been described in patients with AIDS. Epstein-Barr virus is highly associated with CNS lymphoma (Pednaultt *et al.,* 1992). Over 90% of patients with a diagnosis of lymphoma at this site have detectable EBV DNA in their CSF or tumour tissue. This virus has also been associated with ocular inflammation in a variety of patients with EBV-associated systemic disease, although its significance in the ocular disease of HIV-infected patients would appear to be limited. Both HSV 1 and VZV have been described as causes of acute retinal necrosis syndrome and outer retinal necrosis syndrome in patients with HIV-infection, and HHV6 has been co-detected by PCR in the retinal lesions of a patient with active CMV retinitis (Pepose *et al.,* 1984, Jabs *et al.,* 1987b and Margolis *et al.,* 1991). HSV and VZV infections are associated with encephalopathy and peripheral neuropathy in the CNS of HIV-infected patients, either of which may cause significant patient morbidity in those affected. Treatment with ACV

has been found to be highly effective against both HSV and VZV-associated disease in the CNS (Dix *et al.*, 1985, Petito *et al.*, 1986). Isolated cases of HHV6 encephalitis have also been reported, however the extent to which this virus, or its close relative HHV7, infects the CNS and eye, thereby affecting patient health is still largely undetermined (Saito *et al.*, 1995). HHV8 has yet to be described in the ocular fluid of HIV-infected patients, but recently has been found associated with a small number of cases of encephalopathy in severely immunocompromised AIDS patients (Said *et al.*, 1997).

3. e. 2. Study of herpesvirus genome prevalence in post-mortem ocular and CNS samples from HIV-infected patients

In this study material was collected to examine the prevalence of all eight characterised human herpesviruses in the ocular fluid and CNS of a cohort of HIV-infected patients who had died of an AIDS-associated illness. The presence of herpesviral DNA was compared with patient pre-mortem diagnosis and ocular examination, post-mortem anatomical description and post-mortem histological analysis of the ocular and neurological sites. The aims of the study were to compare the accuracy of molecular genome detection techniques with clinical and histological observation, especially with regard to patients with a pre- or post-mortem diagnosis of CMV retinitis and / or encephalitis. It was also hoped that the range and prevalence of the other herpesviruses at these two sites could be determined and an evaluation made as to their associations with pathological disease. This is the first study of such post-mortem material to investigate the presence of all known human herpesviruses and provide correlation with clinical findings.

3. e. 2. i) patient profile and samples recovered

Informed consent was obtained to recover samples from 31 HIV-infected patients at post-mortem. Twenty-nine of the patients were male of whom 26 were homosexual, 2 were intra-venous drug users and 1 an African heterosexual. Both female patients were heterosexual. The median age of patients was 36 years (range 27 - 53 years). In general the level of immunosuppression experienced by this patient group was extremely severe. The range of CD4 counts was 0 - 380, however in all but one patient the CD4 count was below 120. The median CD4 count was just 10.

The samples recovered from each patient were dependent on the consent provided. From patient 1 both eyes were recovered, as was a portion of frontal lobe tissue. From patients 2 - 13 the right eye only was recovered as again was frontal lobe tissue. From the last 18 patients both eyes were recovered, however instead of frontal lobe tissue being sampled a portion of CSF, collected after removal of the brain from the skull, was provided. A total of 100 vitreous and aqueous samples were removed from the cadaver eyes, all within 24 hours of death. These were extracted and processed by the methods described in section 2. c. 7. In the study 10 µl of aqueous and 1 µl vitreous fluid supernatant were analysed for the presence of each herpesviral genome. Frontal lobe tissue was prepared by SDS-protinase K digestion as described in section 2. c. 4. The DNA from this material was not however then subsequently purified by phenol / chloroform extraction instead it was boiled to inactivate the proteinase K and 1µl of a 1 in 10 dilution of the gross preparation analysed. Such a dilution step has previously been shown sufficient for avoiding inhibition of the PCR process by the SDS present in the extracted material (personnel communication, Dr JD Fox, University College Wales School of Medicine). Post-mortem CSF was stored and prepared for analysis in the same manner as pre-mortem CSF, (see section 2. c. 5). Ten microlitres of supernatant was then analysed by each of the herpesviral PCR assays.

3. e. 2. ii) clinical and histological analysis of study patients

All the patients in the study were under the in-patient care of Dr. Rob Miller, UCL Hospitals, a consultant HIV-physician who provided the pre-mortem clinical evaluation. Patients with loss of visual function were referred to a specialist HIV ophthalmic clinic and were reviewed by Dr. Peggy Frith, UCL Hospitals, an ophthalmologist who is experienced in the diagnosis and treatment of HIV-associated ocular disease. All post-mortems were performed by Professor Sebastian Lucas, UCLMS, a specialist with over ten years experience in the post-mortem analysis of HIV-infected individuals. Professor Lucas also conducted all the post-mortem microscopic and histological analysis of removed tissue, and provided the post-mortem clinical evaluation. Histological diagnosis of CMV disease was by visualisation of cellular inclusion bodies and by immuno- and histological antibody staining.

3. e. 2. iii) ophthalmic virology results

Of the 31 patients 8 (26%) had a pre-mortem diagnosis of CMV retinitis with 7 patients having bilateral retinitis and 1 patient unilateral retinitis, a total of 15 eyes with CMV disease (table 3. 8. 1.). Fourteen of these 15 CMV-affected eyes were available for virological analysis. In all 14 eyes CMV DNA was detected in both the aqueous and vitreous fluids. In the one patient (patient 24) with unilateral CMV retinitis both eyes were available, however CMV DNA was detected in the vitreous and aqueous fluids of the CMV-affected left eye only. Six of these 8 patients also had post-mortem evidence of non-ocular CMV disease (5 in the CNS, 1 in the lungs).

Twenty-three patients did not have a pre-mortem diagnosis of CMV retinitis, however two of these patients (patients 15 and 26) had post-mortem, histological evidence of unilateral ocular CMV disease. In patient 15 the left eye was affected, in patient 26 the right eye was affected. These two patients

Table 3. 8. 1. Detection of CMV DNA in post-mortem ocular samples

Patient number	Extent of ocular CMV disease	CMV DNA detected (eyes +ve / eyes tested)	CMV-associated CNS disease	CMV disease at other sites
Patients with pre-mortem CMV retinitis				
1	bilateral	2 / 2	encephalitis	disseminated
6	bilateral	1 / 1 (right)	encephalitis	disseminated
13	bilateral	2 / 2	none	none
23	bilateral	2 / 2	none	lungs
24	unilateral (left)	1 (left) / 2	encephalitis	adrenal gland
27	bilateral	2 / 2	encephalitis	diseminated
28	bilateral	2 / 2	none	none
29	bilateral	2 / 2	encephalitis	none
Patients with post-mortem ocular CMV disease				
15	unilateral (left)	1 (left) / 2	none	none
26	unilateral (right)	1 (right) / 2	none	adrenal gland

Table 3. 8. 1. (cont.) Detection of CMV DNA in post-mortem ocular samples

Patient number	Extent of ocular CMV disease	CMV DNA detected (eyes +ve / eyes tested)	CMV-associated CNS disease	CMV disease at other sites
Patients with no CMV ocular disease but CMV DNA in the eye				
4	none	1 / 1 (right)	encephalitis	adrenal gland and GI tract
7	none	1 / 1 (right)	none	adrenal gland and GI tract
9	none	1 / 1 (right)	none	disseminated
12	none	1 / 1 (right)	none	adrenal gland
20	none	2 / 2	encephalitis	disseminated
21	none	2 / 2	none	adrenal gland
25	none	2 / 2	encephalitis	none

each had both eyes available for analysis but CMV DNA was detected in the vitreous and aqueous fluid of the CMV-affected eyes only. One of these patients also had CMV adrenalitis determined at post-mortem, the other had no evidence of extra-ocular CMV disease.

A total of 21 patients were without a pre-mortem or post-mortem diagnosis of CMV ocular disease, although a number of other ocular pathologies were noted: 4 choroditis, 1 choroditis with cotton wool spots, 2 anterior uveitis, 2 cryptococcus, 1 toxoplasmosis, 1 hyperplastic aracnoid and 1 choroid naevus. Nine patients had eyes considered normal by post-mortem analysis. Of these 21 patients 7 had detectable CMV DNA in at least one eye (table 3.8.1., p. 188). Four of the 7 had only the right eye available for virological analysis, all 4 had CMV DNA detected in both the vitreous and aqueous of those right eyes. Three patients had two eyes available for analysis and all 3 had CMV DNA detected in the vitreous and aqueous fluids of both eyes. In each of these 7 patients, who had no CMV-associated ocular disease, there was post-mortem evidence of CMV disease at non-ocular sites of the body; 2 patients "disseminated" CMV, 2 patients CMV adrenalitis, 2 patients CMV adrenalitis and colitis. Three of the 7 had a direct histological diagnosis of CMV encephalitis.

Fourteen patients had neither CMV DNA detected in their eyes, nor evidence of CMV-associated ocular disease. Four of these fourteen patients had non-ocular CMV disease at post-mortem: 2 in the adrenal glands and 2 described as disseminated.

Cytomegalovirus was by far the most commonly detected herpesvirus in the ocular samples. In all the eyes analysed other herpesviral genomes were detected on only 5 occasions (table 3. 8. 2.); in one patient (patient 12) EBV DNA was detected in the right aqueous fluid, in addition this patient had CMV DNA detected in both vitreous and aqueous fluid of the right eye but no pre-mortem or histological diagnosis of CMV retinitis. This patient also had a post mortem-confirmed CNS B-cell lymphoma. One patient (patient 1), with a pre-mortem diagnosis of bilateral CMV retinitis and disseminated CMV disease,

Table 3. 8. 2 Detection of other herpesviruses in post-mortem ocular fluid

Patient number	Eyes tested	Evidence of ocular disease	Viral DNA detected	Systemic disease at post-mortem
1	both eyes	bilateral CMV retinitis	HSV-2 in left eye vitreous	disseminated KS and disseminated CMV
8	right eye only	none	HHV8 in aqueous and vitreous	disseminated KS and disseminated CMV
12	right eye only	hyperplastic arachnoid	EBV in aqueous	B cell lymphoma and CMV in adrenal gland
14	both eyes	choroditis in both eyes	HHV8 in left eye vitreous	disseminated KS
30	both eyes	none	VZV in left eye aqueous	PCP

had detectable CMV DNA in both the aqueous and vitreous of both eyes. This patient also had HSV-2 DNA detected in the left vitreous fluid. A patient (patient 30) with no remarkable eye pathology had detectable VZV DNA in the aqueous fluid of the left eye, this patient had no systemic evidence of VZV infection, but did have VZV DNA detected in the CNS. Two patients (patients 8 and 14) had detectable HHV8 DNA in their ocular samples. Patient 8 had the right eye available for virological analysis and both the vitreous and aqueous fluid contained detectable HHV8 DNA, this patient had no other herpesviruses detected in that eye which was found by histological analysis to be normal. Patient 14 had both eyes available for analysis and HHV8 DNA was detected in the vitreous fluid of the left eye, the other three ocular fluids from this patient had no detectable herpesviral DNA. This patient had non-specific choroiditis in both eyes. Both patient 8 and 14 had disseminated KS reported as a major cause of death at post-mortem and both patients had detectable HHV8 DNA detected in their peripheral blood prior to death. Patient 8 also had HHV8 DNA detected in the CNS. No HSV-1, HHV6 or HHV7 DNA was detected in any of the ocular samples analysed.

3. e. 2. iv) neurological study results

The detection of viral DNA in the neurological samples in this study was correlated with post-mortem analysis of the cadaver brains, no pre-mortem diagnoses were recorded. As with the ocular samples, CMV DNA was the most commonly detected herpesvirus in the sample set (see table 3. 8. 3.). Of the 31 patients 9 had CNS CMV pathology noted, 8 of these had CMV DNA detected in their CNS. However CMV DNA was also detected in the CNS of 8 of the 22 patients without specific CMV pathology described at this site. Of these 8 patients 6 had CMV DNA detectable in their ocular samples and all had some evidence of systemic CMV disease. The next most frequently detected herpesvirus at the neurological site was EBV. EBV has previously been associated with CNS B-cell lymphoma and 4 patients in this study had a post-mortem diagnosis of CNS B-cell lymphoma, 3 of these 4 had detectable

Table 3. 8. 3. Detection of CMV and EBV DNA in post-mortem CNS samples from 31 patients with AIDS

Patients with	CMV DNA detected	EBV DNA detected
CMV-associated CNS pathology	8 / 8	0 / 8
CMV-associated CNS pathology with CNS lymphoma	0 / 1	1 / 1
CNS lymphoma	2# / 3	2 / 3
Other CNS disease	4# / 13	3 / 13
No CNS disease	2# / 6	0 / 6

all 8 patients with CMV DNA detected in the CNS, in the absence of CMV-associated CNS disease, had other sites of CMV disease noted (5 ocular, 2 adrenal gland and 1 lungs).

EBV DNA in their CNS. Of the other 27 patients with no evidence of CNS lymphoma only 3 had detectable EBV in their CNS.

As was found in the analysis of herpesviral DNA in ocular samples, apart from CMV and EBV which have been well defined as common pathogens in the CNS of HIV-infected patients, other herpesviruses were rarely detected in these patients. No HSV-1, HSV-2, HHV6 or HHV7 was found in any CNS samples. However 4 patients did have detectable VZV DNA in their CNS, 2 of these patients had CMV encephalitis (both with detectable CMV DNA in the CNS) and 2 had no post-mortem evidence of neurological disease. None of these 4 patients had evidence of CNS, or systemic, varicella / zoster disease. One of the 4 (patient 30) was, however, the patient in whom VZV DNA was found in aqueous fluid. HHV8 DNA was found in the CNS of 2 patients, both these patients had disseminated KS and one (patient 8) had also had HHV8 DNA detected in their right eye vitreous and aqueous samples.

The full PCR results from all patient samples are tabulated in the attached Appendix C.

3. e. 3. Investigation of ocular sample inhibition of herpesviral PCRs

After completion of the herpesviral analysis on the ocular samples in this study vitreous samples with at least 0.5 ml remaining and aqueous samples with at least 200 µl remaining were used to determine the possible inhibitory effects these samples may have on the PCR process. Inhibition of PCR by factors within ocular fluids, especially vitreous fluid, has been previously described (Wiedbrauk *et al.*, 1995) and could potentially result in a failure to detect clinically important viral genomes. All selected samples had been shown in the study to be negative for CMV DNA, as CMV was to be the control genome used in this analysis. Before use each sample was re-tested, by the CMV PCR, at input volumes of 0.1, 1.0 and 10 µl to confirm the original PCR result.

A ten-fold serial dilution of control CMV DNA was prepared in water to determine its CMV DNA titre in ideal amplification conditions. The lowest dilution detected was given the arbitrary concentration of a single CMV genome as the CMV PCR has previously been shown capable of single-copy detection (Mitchell *et al.*, 1994). The same dilution series was then prepared using each of the ocular fluids as diluent and the input titre required for the production of an amplification product determined. Three different assay formats were investigated using these spiked samples:

A - 1 µl of each spiked ocular fluid analysed in a 50 µl volume 1st round PCR

B - 10 µl of each spiked ocular fluid analysed in a 50µl volume 1st round PCR

C - 10µl of each spiked ocular fluid analysed in a 100µl volume 1st round PCR

All the second round PCR's were performed, as usual, in a 25µl volume reaction, with a 1µl transfer of the first round product to prime the second round reaction. In total 11 vitreous and 21 aqueous samples were analysed by this protocol, results are shown in table 3. 8. 4.

The results in table 3. 8. 4. clearly show the inhibitory effects of increasing the volume of vitreous fluid analysed from the 1 µl used in the study to 10 µl, if a 50 µl 1st round PCR volume is employed. Of the 11 vitreous samples analysed 6 caused some form of inhibition in this format. Three of these 6 samples resulted in a 3 $\log_{10}$ drop in PCR detection sensitivity. Importantly, none of the vitreous samples were, however, inhibitory if only 1 µl was analysed in a 50 µl reaction volume and, equally significantly, all inhibitory effects of analysing 10 µl of vitreous fluid were overcome if the first round reaction volume was increased to 100 µl.

Table 3. 8. 4. Inhibitory effects of ocular fluids on CMV PCR amplification

Ocular fluid	CMV DNA copies required to produce CMV amplicon (in distilled water = 1)		
	PCR format A	**PCR format B**	**PCR format C**
Vitreous-			
1:	1	1	1
2:	1	1	1
3:	1	1000	1
4:	1	1	1
5:	1	100	1
6:	1	10	1
7:	1	1	1
8:	1	1000	1
9:	1	1000	1
10:	1	100	1
11:	1	1	1
Aqueous-			
1 - 20:	1	1	not tested
21:	1	10	1

As has been previously described aqueous fluid seems to contain fewer PCR inhibitors than vitreous fluid. Only 1 of the 21 samples analysed caused any reduction in PCR sensitivity when 10 µl of aqueous was analysed in a 50 µl reaction volume, when this sample was tested in the other two formats no inhibition was noted.

3. e. 4. Discussion

Most of the HIV-infected patients analysed in this study had homosexual contact as their primary risk factor for HIV-1 acquisition. It is this group of patients which have been previously shown to be at greatest risk of developing herpesvirus infections during the course of their HIV disease, especially CMV-infection. This patient group have also been shown to be infected with the greatest range of herpesvirus infections and the greatest number of multiple infections with different strains of the same herpesvirus, especially CMV (Levine, 1993 and Drew *et al.*, 1984). They were therefore an ideal group of patients in which to determine the significance of herpesviral ocular and CNS infection in a severely immunocompromised population.

In the eyes and CNS of these patients there was, apart from the well described associations between CMV and encephalopathy / retinitis, and EBV and CNS lymphoma, a surprising lack of detectable herpesvirus genomes given the highly sensitive detection methodology used. Of the six other herpesviruses only HHV8 and VZV were found in eyes or CNS of more than one patient, and both of these viruses were not associated with any particular disease process. All but one patient with a diagnosis of CMV retinitis or encephalopathy had detectable CMV DNA at those disease sites underlining the clear association between the presence of CMV-related disease and CMV genome. However a significant minority of patients also had detectable CMV DNA in the brain or eye in the absence of overt CMV disease, although most did have some other form of systemic CMV disease. Detection of CMV DNA

in the absence of disease may either reflect contamination of the post-mortem material by CMV DNA-containing blood, or the spread of virus from other body sites. It was interesting to note that all but one patient with detectable CMV in the brain or eye had CMV disease described somewhere in the body. This presence of CMV DNA may have been a precursor to the development of CMV-associated disease and previous studies have shown that detection of CMV DNA in the eye and brain, in the absence of disease, is predictive of subsequent disease development (Cinque *et al.*, 1996a and van der Meer *et al.*, 1996). The finding of CMV DNA in the absence of histological evidence of CMV pathology should, however, underline the fact that detection of herpesviral genome in such severely immunosuppressed individuals is not always indicative of an actual disease involvement.

As well as confirming the established association between CMV and neurological and ocular disease in late-stage AIDS patients this study also supported the association between EBV and CNS lymphoma. Three of the 4 patients with a clinical diagnosis of CNS lymphoma had detectable EBV DNA in their CNS, whereas only 3 of the other 27 patients had this viral genome detectable. The presence or absence of pre-clinical CNS lesions in these 3 patients was not described, but again studies have shown that the detection of EBV DNA in the CSF is predictive of CNS lymphoma development in such patients (Pednaultt *et al.*, 1992 and Arribas *et al.*, 1995).

It was possible to confirm that some ocular samples, when crudely prepared as here, may have significant inhibitory effects on the sensitivity of the herpesviral nested PCR assays. A significant proportion of the vitreous samples analysed reduced assay sensitivity by up to 3 $\log_{10}$ when 10 µl was analysed in a 50 µl 1st round reaction volume but a 1 µl volume, as used in the study, was not found to be inhibitory. The effects of these inhibitors was also negated when the reaction volume was increased to 100 µl. As early detection of CMV DNA in the vitreous fluid is of pronounced clinical significance maintaining assay sensitivity, while analysing the greatest volume of material possible, is

paramount. The use of a 100 µl first round reaction volume, allowing a 10-fold increase in overall assay sensitivity, by analysis of 10-fold more vitreous fluid is, therefore, of significance in the routine diagnostic detection of herpesviruses in the eye. Aqueous fluid was found to be rarely inhibitory to the nested PCR even at a 10 µl input volume in a 50 µl first round reaction volume.

In conclusion it would appear that the CNS, including the eye, is a sub-optimal environment for the infection and replication of the majority of herpesviruses, a finding which contrasts with the concept of the brain being an immunologically privileged site, and the high prevalence of previous herpesvirus infection in such a patient group. Although there have been some reports of HSV and VZV causing CNS, and ocular, disease their "index of suspicion" for a clinician when confronted with a patient experiencing symptomatic disease at these sites must be low and they were not found associated with disease in this patient group. HHV6 and 7 were not detected in the patients tested and again would appear to be of little clinical importance even though most, if not all, patients are likely to have been infected. There is limited previous data regarding the association of HHV8 and ocular and neurological disease. The HHV8 DNA detected in the brain and ocular samples in this study was unassociated with specific disease and KS is a rare complication of the retina or CNS, although it has been found to involve the conjunctiva in a small number of KS patients. In a similar manner to the CMV DNA detected in patients without CMV pathology, the detection of HHV8 may simply reflect the presence of systemic HHV8 DNA in the 3 patients involved, or the occurrence of pre-clinical ocular or neurological lesions not noted at post-mortem. It should, however, be remembered that the close genetic relative of HHV8, EBV, does cause significant CNS disease so an association of HHV8 with CNS damage cannot be completely discounted.

Overall these findings confirm a significant association between encephalitis, retinitis and CMV, and between CNS lymphoma and EBV. These two

herpesviruses are the most clinically important in the CNS and eyes of late-stage AIDS patients and hence should be the principle focus of molecular diagnostic assays such as PCR. Clinical therapeutics should be directed accordingly. This is especially important as early treatment for both CMV and EBV-associated CNS / ocular disease has been shown to be highly effective in moderating disease and in delaying associated mortality (reviewed in Drew, 1992 and Donahue *et al.*, 1995).

3. f. Evaluation of a nested PCR multiplex assay for the detection of six clinically important herpesvirus genomes.

3. f. 1. Introduction

Of the eight characterised human herpesviruses, six have consistently been found associated with significant disease in immunocompromised patients: HSV-1, HSV-2, VZV, EBV, CMV and HHV-8. Since 1992 individual nested PCR's have been available for detection of 5 of these 6 viral genomes in the Department of Virology, UCLMS (Mitchell *et al.,* 1994). The sixth, HHV8, has been available since 1995 (Howard *et al.,* 1997). Patients for whom rapid, molecular-based diagnosis of herpesvirus infections is most critical include those receiving immunosuppressive therapy following allogeneic bone marrow transplantation, patients with AIDS and neonates presenting with acute neurological disease (Aslanzadeth and Skiest, 1994, Mitchell *et al.,* 1994 and Woo *et al.,* 1997).

At the end of 1995 an internal audit was commissioned in order to evaluate the use of molecular diagnostic techniques within the Department of Virology, UCLMS. Over the three month period analysed it was found that approximately 1200 herpesvirus analysies were requested on 352 clinical samples. However only 75 herpesviral genomes were actually detected by the individual PCR's. With such a low prevalence (21%) of herpesviral genome in the samples received by the Department it was felt necessary to modify the genome detection protocol so that negative samples could be rapidly excluded while samples containing genome would continue to be reliably detected. The most rational solution to this situation was felt to be the development of a nested multiplex PCR (mPCR) reaction with subsequent detection of amplicons by an enzyme-linked oligonucleotide assay (ELONA).

The method for the mPCR-ELONA is described in section 2. f.. Briefly in the mPCR primers for amplification of all six target herpesvirus genomes are made

available within a single reaction mix. The reaction consists of two rounds of amplification. The visualisation of the amplified mPCR products is by the capture of the second round amplicons, via covalently linked 5' biotin molecules, onto a streptavidin-coated solid phase. The captured amplicons are then single-stranded by the addition of sodium hydroxide before incubation with a pool of six amplicon-specific, enzyme-linked, oligonucleotide probes. Each of the six probes used is specific for one species of amplified herpesviral DNA. Hybridisation of a probe to the solid-phase bound mPCR product results in presentation of a horse-radish peroxidase (HRP) molecule. When incubated with the substrate TMB a colorimetric reaction results which, after addition of 2N H_2SO_4 to stop the reaction, allows differentiation between positive and negative mPCR reactions by analysis at 450nm using a spectrophotometer. Samples found to contain herpesvirus DNA by this "all-6 probe" screen are then re-analysed using each of the herpesvirus-specific probes individually to define virus type.

The mPCR-ELONA assay was previously optimised and its absolute detection sensitivity determined in collaboration with Mr. Stuart Kirk, Department of Virology, UCLMS.* The overall sensitivity of the assay was found to be the same as for the individual PCR assays previously used (Mitchell *et al.*, 1994, Howard *et al.*, 1997). In general this was equivalent to a single target genome molecule. The six HRP-oligonucleotide probes were each found specific for their specified mPCR amplicons and no cross-reactivity was noted.

* ached to inside back cover of this thesis

3. f. 2. Analysis of single copy herpesviral genome molecules using the mPCR-ELONA assay.

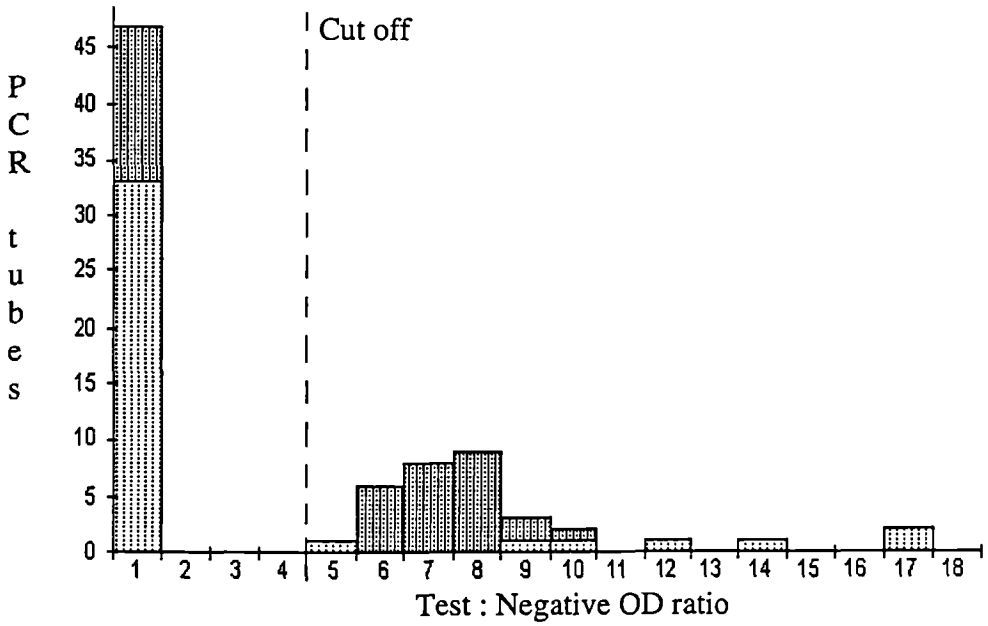
Following optimisation of the mPCR-ELONA assay using control genome samples and comparison with the individual PCR assays the positive cut-off of the assay was set at five times the average of the negative control samples; the test : negative ratio. In practice experience showed that the OD of a negative control sample was usually between 0.12 and 0.17, therefore a test sample was

only defined as containing detectable HHV8 DNA if the OD it produced was approximately 0.9 or above. To ensure that the assay was reproducibly capable of single copy genome detection, as had been suggested by comparison of end-point titration sensitivity with the individual PCR's, its behaviour when analysing mixtures containing single target molecules was investigated. Two solutions containing low copy numbers of CMV and HSV-1 DNA were separately prepared by ten-fold serial dilution of purified genomic DNA. To determine the exact genome copy number of each of these solutions 1µl from each was diluted in 399µl water and the total 400µl divided equally between 40 CMV and 40 HSV-1 PCR assay tubes. Conventional individual PCR analysis was then performed for these two targets. Fourteen of the CMV DNA PCR tubes produced amplicons when analysed by gel electrophoresis, as did 3 of the HSV-1 DNA PCR tubes. This determined the detectable genome copy numbers, per microlitre, of each genome in their respective solutions.

The analysis was then repeated on 1µl of these two DNA solutions by 40-tube analysis using the mPCR-ELONA assay with HRP-probes for detection of CMV and HSV-1 amplified products. After analysis of the ELONA results the negative control reactions gave an average OD of 0.13. The amplicons from 26 tubes analysed with the CMV-specific probe and 7 analysed with the HSV-1 probe resulted in ELONA OD's greater than 5x these average negatives (OD range 0.69 - 2.22, test : negative ratio 5 - 17, fig. 3. 9. 1.). The remaining tubes all resulted in OD's less than 5x the average negatives (OD range 0.12 - 0.18, test : negative ratio 0.92 - 1.4, fig 3. 9. 1.).

These results suggest that the mPCR-ELONA can indeed reliably detect single-copy molecules and that the 5x negative average cut-off is appropriate, even when analysing such small amounts of target sequence. There was some difference in the number of genomes detected in the 1µl of control genome solution between the two assays: 14 CMV DNA copies in the individual PCR, in contrast to 26 copies in the mPCR-ELONA, and 3 HSV DNA copies in the individual PCR, in contrast to 7 in the mPCR-ELONA, however with the

Figure 3. 9. 1. Optical densities derived from 80 tube analysis, by mPCR-ELONA, of single copy CMV and HSV genomes prepared by Poisson Distribution analysis.



OD signals from theoretical single-copy CMV genomes, 26 genomes detected in 40 tubes by mPCR-ELONA, (CMV PCR detected 14 genomes in 40 tubes).

OD signals from theoretical single-copy HSV genomes, 7 genomes detected in 40 tubes by mPCR-ELONA, (HSV PCR detected 3 genomes in 40 tubes).

stochiometry of analysing DNA molecules in a solution at the level of Poisson distribution this is not unusual and does not suggest a significant difference in sensitivity between the two assay formats.

3. f. 3. Detection of herpesviral genomes in 192 clinical samples using the mPCR-ELONA.

To determine the overall sensitivity and specificity of the mPCR-ELONA assay in comparison to the existing individual PCR reactions a blinded panel of 192 clinical specimens was analysed. The samples used (table 3. 9. 1.) were stored and processed as described in section 2. c. All the samples used were received into the Department of Virology, UCLMS for routine herpesvirus analysis and had previously had the presence or absence of herpesvirus DNA determined by the individual PCR assays. Samples were defined as containing detectable herpesviral genome if amplification had occurred in at least 2 separate individual PCR analyses. The majority of samples used in the evaluation were CSF as this analyte is rarely found to contain any inhibitory factors which might affect the efficiency of the mPCR. Analysis of the samples by the mPCR-ELONA was done blinded to the results of the individual PCR assays.

Seventy of the 192 samples had been found, by individual PCR, to contain herpesvirus genomes. In total, 81 individual herpesvirus genomes were detected in these 70 samples (11 samples contained two detectable genomes). 66 (94%) of the 70 samples were also found to have detectable herpesvirus genome when analysed by the mPCR and the 6-probe "screening ELONA" which detects the presence of any of the 6 herpesvirus genomes. Individual ELONA-probe typing of the 66 mPCR-positive samples showed exact concordance with the individual PCR analysis for every sample, including the detection of 2 separate genomes in all samples where 2 genomes had been detected by the individual PCR's. Overall 77 (95%) of the 81 PCR-detectable herpesviral genomes in the clinical panel were correctly identified and typed by

Table 3. 9. 1. Clinical samples tested by multiplex PCR-ELONA

Sample type	Total number tested	Herpesviral DNA not detected by individual PCR	Herpesviral DNA detected by individual PCR
CSF	118	102	16
Tissue culture isolates	32	12	20
Vesicle fluid / swab	10	2	8
Bronchio Alveolar Lavage fluid	7	3	4
Whole blood (QIAGEN)	6	1	5
Extracted white blood cells (glycigel)	8	0	8
Mid stream urine	4	0	4
Biopsy tissue	3	3	0
Ocular:			
Vitreous fluid	2	0	2
Aqueous fluid	1	1	0
Plasma	1	0	1
Totals	**192**	**122**	**70**

the mPCR-ELONA assay (table 3. 9. 2. and figure 3. 9. 2.). The test : negative ratio OD's generated from these mPCR-ELONA positive samples ranged from between 5 - 24 times the average of the control negatives.

There were 4 samples in which herpesvirus DNA had previously been found by the individual PCR, but was not detected by the mPCR "screening-ELONA." Three of these samples, all CSF's, had sufficient remainder to be reanalysed by the individual PCR's and were confirmed as containing the genomes originally detected (table 3. 9. 3.). The detectable genome copy numbers of each sample were then defined by end-point titration analysis and in all three cases found to be extremely low, at 1 copy per microlitre or less. The fourth sample had insufficient volume remaining for retesting.

None of the 122 samples shown to have no detectable herpesviral DNA by the individual PCR assays were found to have detectable herpesviral DNA by the mPCR "screening-ELONA" (table 3. 9. 2.). The test : negative ratio OD's produced from all of these samples were well below the 5x cut-off at between 0.7 - 1.4 (figure 3. 9. 3.).

3. f. 4. Discussion

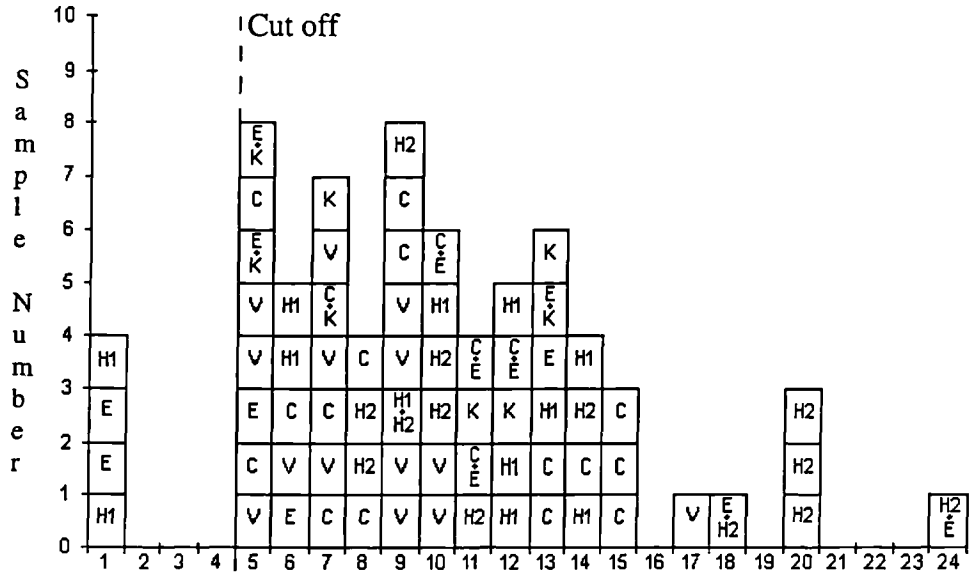
Over recent years a number of methods have been proposed by which PCR may be utilised for the simultaneous detection of diverse herpesvirus genomes. These have included the development of universal primers against highly conserved viral gene sequences, the use of multiple primer sets in multiplex reactions with electrophoretic separation of amplification products and the use of restriction enzyme digestion to differentiate multiplex PCR products of similar size by their fractionation patterns (Chenel and Griffais, 1994, Orle *et al.*, 1996 and Roberts and Storch, 1997) . Each type of methodology has suffered from a variety of weaknesses, including a lack of detection sensitivity and a limit to the number of species able to be analysed (Cassinotti *et al.*, 1996).

Table 3. 9. 2. Correlation of results obtained between individual nested PCR assays and multiplex PCR-ELONA

	Multiplex PCR result											
	HSV-1		HSV-2		VZV		CMV		EBV		HHV8	
Individual PCR result	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg
Positive	10	2*	13	0	14	0	20	0	12	2*	8	0
Negative	0	126	0	127	0	81	0	85	0	72	0	50

* discordant results between individual PCR assay and multiplex assay (see text for full explanation)

Figure 3. 9. 2. Test : Negative optical densities from mPCR-ELONA "all-probe" detection of 70 samples shown to contain at least 1 herpesvirus genome by individual PCR assay analysis



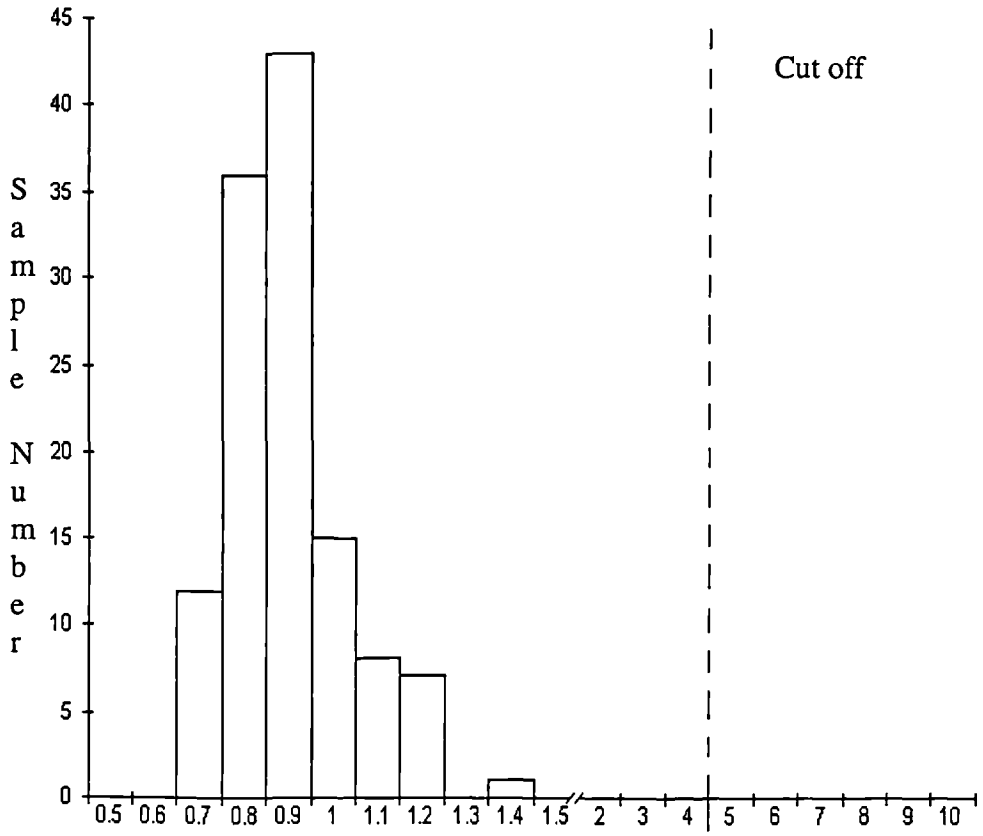
Uppercase letters indicate herpesviral genomes detected in each sample by individual PCR assay (H1 = HSV-1, H2 = HSV-2, V = VZV, C = CMV, E = EBV and K = HHV8).

Table 3. 9. 3. Four CSF samples in which herpesvirus DNA was detected by individual PCR assays but not by mPCR-ELONA.

Herpesvirus DNA detected by individual PCR assay	Herpesvirus genome load in samples	Test : Neg OD ratio of sample by mPCR (+ve cut-off = 5.0)
HSV-1	**0.1 copies/µl**	1.4
EBV	**1 copy/µl**	1.2
EBV	IS	1.3
HSV-1	**0.1 copies/µl**	1.0

IS = Insufficient sample for quantification analysis.

Figure 3. 9. 3. Test : Negative optical densities obtained by mPCR-ELONA all-probe detection of 122 samples previously found not to contain detectable herpesvirus DNA by individual PCR analysis.



The mPCR-ELONA assay evaluated in this chapter appears to have overcome both these restrictions through the use of three separate hybridisation reactions (nested PCR primers followed by an internal oligonucleotide probe) which has allowed reaction conditions to be chosen which maintain the specificity and sensitivity of the assay compared to the previously-used individual PCR assays. Overall a 95% correlation was found between the two formats in the analysis of samples containing herpesviral DNA, this correlation increases to 98% when samples found not to contain any detectable herpesvirus genome are included in the calculation. Such a high level of agreement between the two assays has led to discussions within the Department of Virology which should soon result in the use of the mPCR-ELONA as our herpesvirus molecular diagnostic assay of choice.

An analysis of the individual nested PCR assay shows an approximate cost, per sample, of £4 (this figure includes the requirement for control reactions and all reagents, but does not include the cost of DNA preparation). For analysis in the mPCR-ELONA the cost is slightly higher at approximately £6 (including both the "screening-ELONA" and subsequent type-specific analysis). However the 1995 audit showed that, on average, each sample received has investigation for 4 herpesvirus genomes requested. Not only does this greatly increase the time taken to provide the service using individual PCR (the mPCR can provide the same service with a 65% reduction in the number of assay reactions required) but also raises the financial cost of individual PCR analysis to £16 per sample. As a large percentage of clinical samples are found to contain no herpesvirus genomes the most significant fiscal benefit of the mPCR-ELONA must be the reduction in cost required to effectively screen out negative samples.

Whilst showing a very high level of concordance with the individual PCR assays the mPCR-ELONA did fail to detect to detect herpesvirus DNA in 4 samples. Three of the 4 samples were reanalysed and all were found to contain very low levels of target DNA. Such a finding suggests that the mPCR-

ELONA may be slightly less sensitive than the individual assays, although this is unlikely as in the single target molecule analysis the sensitivity of the two formats was found to be the same. It is possible that these 4 samples contained a level of PCR inhibitors which adversely affected the mPCR-ELONA but not the nested PCR's. It was unfortunately not possible to determine the presence of inhibitors in these samples due to their limited volume. As mentioned earlier CSF samples were chosen for the majority of the evaluation because they are usually free from biological inhibitors, other samples analysed were all purified prior to analysis. One simple solution to the problem of inhibitors affecting PCR amplification would be to adapt the processing of the CSF samples so that a purification step was included, probably similar to the Qiagen protocol used for the preparation of whole blood (see section 2. c. 1.). However this would increase the work load and cost of sample analysis. The alternative is to include, within the mPCR-ELONA, a low copy-number control genome which is added to all samples prior to the mPCR and then must be detected in the "screening-ELONA" to confirm amplification from that sample. Such a control genome protocol is currently being investigated within the Department of Virology. The genome chosen is murine and would be added to each clinical sample preparation at a level of 10 detectable copies. As soon as this addition to the assay is available the CSF samples in the clinical panel found not to contain any herpesvirus DNA will be spiked with the murine genome to determine the prevalence of any inhibitors. Should such inhibitors be common in these samples further purification will then be required, if not the murine genome will continue to be used in the assay as an additional intra-sample control feature.

Section 4: General Discussion

4. a. HHV8 infection in the human host

4. a. i) Disease associations

Since its original discovery in KS tissue from an HIV-infected individual (Chang *et al.*, 1994), HHV8 DNA has been consistently detected in approximately 90% of all types of KS lesions (Knowles and Cesarman, 1997). This includes endemic KS (Rady *et al.*, 1995a and Boshoff *et al.*, 1995), iatrogenic KS (Alkan *et al.*, 1997) and classical KS (Dictor *et al.*, 1996). These early reports of viral prevalence related to the detection of viral DNA in the KS tumour mass, subsequent studies, including those described in section 3. a. 4., have shown that the HHV8-KS aetiologic link is maintained when the prevalence of HHV8 viraemia is investigated and that the presence of HHV8 DNA in blood is significantly associated with a clinical diagnosis of KS (Whitby *et al.*, 1995 and Moore *et al.*, 1996). Of even greater significance is the increased probability of HIV-infected patients, without KS, to develop KS if HHV8 is detected in their blood, even though a delay of months, or years, may exist between detection of the virus and development of disease. In section 3. a. 5. it was shown that this predictive association was maintained when a specific form of KS was examined, as 5 of 5 HIV-infected patients developed the pulmonary form of KS within 18 months of HHV8 DNA being detected in lavage fluid from their bronchial tree.

The association between HHV8 infection and KS has been underscored by the use of serological assays for the detection of anti-HHV8 antibodies. Whereas only 1-5% of HIV-negative UK heterosexuals have evidence of HHV8 infection

(Simpson *et al.*, 1996), the infection rate rises to 30-50% in areas in which HIV-unassociated KS is more common (Gao *et al.*, 1996) and is over 80% in patients with an actual diagnosis of KS (Kedes *et al.*,1996). With the provision of improved serological reagents the prevalence of anti-HHV8 antibodies is expected to rise to nearer 100% in those patients with KS. When the prevalence and epidemiology of HHV8 is considered, together with the recent identification of HHV8 gene products which may be involved in cell cycle dysregulation, such as the viral homologues to cellular Il-6 (Neipel *et al.*, 1997), type-D cyclin (Cesarman *et al.*, 1996) and Bcl-2 (Chang *et al.*, 1996) it now appears that an overwhelming weight of evidence supports a role for HHV8 in the development and maintenance of KS tumours. This is not to say that HHV8 is the only requirement for the genesis of KS lesions. As has been noted for a variety of other virus-related cancers it is usually a range of factors which contribute to tumour formation. One need only consider the differing forms of EBV-associated tumours to appreciate the effect that other infectious agents, environmental factors and the state of the host immune system may have on neoplastic development.

In addition to KS HHV8 has been associated with a number of other diseases, most of which mainly occur in the HIV-infected population. The most secure of these associations is with the body-cavity-based lymphomas (Cesarman *et al.*, 1995 and Gessain *et al.*, 1997). These rare pleural malignancies have been consistently found to contain HHV8 DNA, and whole viral particles, while no HHV8 DNA is detected in other forms of HIV-associated and unassociated lymphoma (Cesarman and Knowles, 1997). Indeed the HHV8-infected immortalised B-lymphocytes used in section 3. c. of this thesis, for the detection of anti-HHV8 IgG by immunofluorescence assay are derived from BCBL effusions (Gao *et al.*, 1996 and Boshoff *et al.*, 1998). The probable mechanisms of HHV8-induced cell transformation, again through the expression of gene products mimicking cell cycle control proteins, is well described (section 1. a. 8.

and Boshoff *et al.*, 1998) and supports the activity of the virus in BCBL development.

The third disease showing a significant association with HHV8 is multicentric Castleman's disease (Soulier *et al.*, 1995 and Corbellino *et al.*, 1996). Before the discovery of HHV8 it had been reported that patients with MCD had an increased likelihood of developing KS, approximately 13% of HIV-uninfected patients and 75% of HIV-infected patients with MCD were noted to have subsequently developed KS (Peterson and Frizzera, 1993). The presence of HHV8 DNA in almost all HIV-associated MCD lesions and in over half of the HIV-uninfected patients with MCD is therefore unsurprising, and implies a role for HHV8 in this disease.

A variety of other cutaneous lesions, including malignant endothelial lesions, angiosarcoma and endothelial haemangio-sarcoma, all of which have a histological resemblance to KS, and involve similar cell types, have been investigated for the presence of HHV8 DNA. Boshoff and colleagues examined 37 squamous carcinoma samples (Boshoff *et al.*, 1996) from immunocompromised patients, in response to a study by Rady and colleagues which showed some association between skin cancer in the immunocompromised host and HHV8 (Rady *et al.*, 1995), however none of these 37 carcinoma samples were found to contain HHV8 DNA. More recently Cathomas and colleagues found no HHV8 DNA in 30 epithelial skin tumours and non-Hodgkin's lymphoma samples (Cathomas *et al.*, 1997) and Chang and colleagues (Chang *et al.*, 1996a) and Kenemy and colleagues (Kemeny *et al.*, 1996), were unable to detect HHV8 DNA in a large number of benign angiogenic lesions. HHV8 DNA has been detected in 4 angiolymphoid hyperplasia (Gyulai *et al.*, 1996), a venous haemangioma (Herman *et al.*, 1998), as well as 7 angiosarcomas (McDonagh *et al.*, 1996), however the evidence currently available would suggest that such associations are still unproven.

Apart from KS, BCBL and MCD another disease with which HHV8 infection has been linked is multiple myeloma. Such an association was first suggested by Rettig and colleagues (Rettig *et al.*, 1997), based upon the detection of HHV8 DNA and transcription products in dendritic and transformed plasma cells from patients with MM, and its precursor condition MGUS. A number of other studies which investigated this proposed association were, however, unable to confirm these findings (Parravicini *et al.*, 1997, Masood *et al.*, 1997 and Whitby *et al.*, 1997). In this thesis (section 3. c.) a serological and genomic analysis was conducted for evidence of HHV8 infection in a cohort of patients with haematological malignancies, including 6 with MM. The prevalence of HHV8 in this cohort (8%) was little different to the UK average of approximately 5% and HHV8 infection was not associated with any particular malignancy. Until further evidence, such as that supplied for the association between HHV8 and KS is forthcoming, argument will continue as to the validity of Rettig's claims and therefore any association between HHV8 and myeloma must, at present, remain suspect.

4. a. ii) Diagnostic value of HHV8 DNA and anti-HHV8 IgG detection

The clinical value of detecting HHV8 DNA in tissue from a patient with cutaneous KS is limited as histological examination of the tumour remains a highly accurate method for making a definitive diagnosis of KS (reviewed by Levine, 1990). The value of detecting HHV8 DNA in the blood of HIV-infected patients without KS may, however, be of greater importance. As such detection appears predictive of subsequent KS development individuals with an HHV8 viraemia may be monitored more closely for the development of early KS patches. At a very early stage such lesions respond well to local cryotherapy, or radiotherapy and may also be treated with non-cytotoxic systemic

agents, including thalidomide, as described in section 3. b.. The presence of HHV8 DNA in blood may also be useful in promoting the detection of KS tumours presenting at non-cutaneous sites, such as the oral cavity and lymph nodes (Yokois *et al.*, 1997 and Ficarra *et al.*, 1988), where KS may initially go unnoticed by both the patient and clinician.

In terms of health education defining the presence of HHV8 DNA in the semen or blood of a gay man, or the detection of antibodies to the virus, would be of benefit in advising the patient of the transmission risk to uninfected partners. Since the advent of the HIV-epidemic education in the prevention of viral transmission through sexual contact, especially in the homosexual population, has been dramatically improved. However there remain many instances in which partners are either both HIV-infected, or uninfected, and refrain from using normal barrier methods of protection. As HHV8 DNA may be detected in the semen of gay men both with, and without, KS (section 3. a. 6.) it is highly probable that sexual transmission of this virus, like CMV, occurs in the homosexual population. Epidemiological studies of KS incidence (Beral *et al.*, 1990 and 1993) and serological studies of HHV8 infection in gay men (Martin *et al.*, 1998) support this theory of sexual transmission and underline the importance of avoiding unprotected intercourse between HHV8 infected and uninfected partners.

The particular clinical situation in which detection of HHV8 DNA is of significant benefit is in the bronchial washings from patients at risk of developing pulmonary KS. Of all forms of KS, pulmonary KS has the highest associated rates of patient mortality and is often only detected at post-mortem (Zibrak *et al.*, 1986). It is also patients with pulmonary KS which benefit most from prompt therapeutic intervention with systemic cytotoxic treatment, especially liposomal daunorubicin (Shurmann *et al.*, 1994). The study in section 3. a. 5., as well as that by Tamm and colleagues (Tamm *et al.*, 1998), showed that over 80% of patients with pulmonary KS have detectable HHV8 DNA in their

pulmonary cavity. Detection of the virus at this site is, therefore, an excellent additional diagnostic marker of pulmonary KS involvement. If found in patients with non-specific respiratory disease such detection should focus investigation on the heightened possibility of KS lesions being present in the bronchial tree. The study in section 3. a. 5. also showed the predictive value of detecting HHV8 DNA in the lungs of patients with, or without, cutaneous KS. Although no specific therapy to prevent the development of pulmonary KS lesions in such patients is currently offered the finding of pulmonary HHV8 DNA increases the probability that future respiratory disease may have a KS involvement (Howard *et al.,* 1998 and Tamm *et al.,* 1998).

Apart from use in populations known to be at increased risk of developing HHV8-associated disease the value of diagnostic techniques for HHV8, especially serological analysis for anti-HHV8 antibodies, would be in the screening of blood and tissue donors. The studies in section 3. a. 4 and 3. a. 7 suggest that the risk of acquiring HHV8 in the UK, through the receipt of donated blood or semen is very low, especially as homosexual men are already prevented from being donors because of the increased risk of HIV-1 transmission. Therefore it is unlikely that all donors would require screening for HHV8 infection, however it would appear sensible to ensure that donations being used to treat immunocompromised individuals, who are known to be at an increased risk of HHV8-associated disease, are derived from HHV8-seronegative donors. Such a protocol has already been shown to be highly effective in the prevention of CMV disease in immunocompromised transplant patients (Martin *et al.,* 1990 and Eastlund, 1995) This was confirmed by the study of allogeneic bone marrow transplantation, described in section 3. d., where none of the CMV-seronegative recipients of bone marrow or blood products from similarly CMV-seronegative donors had evidence of systemic CMV infection post-transplant.

4. a iii) Possible treatments for Kaposi's sarcoma

KS remains the most common malignancy in the HIV-infected population, with 30% of all HIV-infected gay men developing KS during their HIV-associated illness (Peters *et al.,* 1991). Over the last 15 years a variety of therapies for KS have been investigated, most with limited success. Recently a combination of strategies have been proposed. For severe, systemic (especially pulmonary) KS cytotoxic chemotherapeutics, such as the angiogenesis inhibitors (e.g. liposomal daunorubicin) can be effective and are clearly indicated in patients with progressive disease (Harrison *et al.,* 1995). Whilst being effective in the treatment of advanced KS such therapies have significant associated toxicity's and are impractical for use in patients with early, or limited cutaneous KS. In such patients the traditional approach has been the use of local radiotherapy, or cryotherapy (Volm and Roenn, 1995). This has a cosmetic value in reducing the size and pigmentation of individual lesions but has no effect in delaying or reducing the development of additional lesions, or moderating the systemic spread of the disease. For this reason a variety of alternative therapies, such as the interferon family of compounds and systemic retinoids, (Harris *et al.,* 1992 have been investigated. Unfortunately clinical response rates to these treatments have been mixed, side effects are common and none have found widespread use. Such therapies also rely on the patient having a degree of remaining immune function. As described in section 3. 4. an alternative therapy for the treatment of early cutaneous KS is oral thalidomide. With its low-levels of associated side-effects and simple administration regime thalidomide was shown to be highly active against KS in the patients who completed an eight week course of therapy. As well as providing a partial clinical response rate of over 60% the levels of systemic HHV8 DNA in patients undergoing treatment were greatly reduced, indeed in most patients viral DNA became undetectable in peripheral blood. As previously discussed it was impossible to determine whether such viral clearance was a direct effect of the drug on HHV8, or an indirect effect due to the

reduction in tumour load. However the clinical results of this study and one other conducted at the National Institute of Health, USA (Welles *et al.*, 1997) suggest that thalidomide may be a valuable treatment option in a patient group currently given little therapeutic support for their KS. The response of HHV8 DNA titre in the patients treated with thalidomide, both in those who responded to treatment and those who had progressive disease, also highlights the possibility of using systemic HHV8 load as a criteria for evaluating clinical response to other anti-KS therapies.

Two other types of therapy have been suggested for the treatment of KS. The first of these are the established anti-herpesviral agents, such as PFA and GCV. Little information is currently available as to the effectiveness of such treatments for KS. One study (Humphrey *et al.*, 1996) of five patients showed a persistence of systemic HHV8 DNA during intravenous administration of PFA and GCV. HHV8 has, however, been found by *in vitro* study to be sensitive to both GCV and PFA (Medveczky *et al.*, 1997 and Kedes and Ganem, 1997). The other group of chemotheraputics which have been shown to be effective in the management of all forms of HIV-associated KS are the anti-HIV protease inhibitors. The regression of iatrogenic KS is well described in non-HIV infected immunocompromised patients who have their immune function restored (Sangiorgi *et al.*, 1993). This suggests that debilitation of the host immune system is crucial in the development and maintenance of KS. Therefore, probably because of their significant immune system-enhancing effects, a number of centers have reported a dramatic response in their patient's KS after use of the protease inhibitors Indinovir and Ritonovir (Krischer *et al.*, 1998 and Lebbe *et al.*, 1998). Both thalidomide and PFA also have some reported anti-retroviral activity (Moreira *et al.*, 1997 and Devianne-Garrigue *et al.*, 1998) and it may be that their effectiveness in the treatment of HIV-associated KS is also, in part, be due to an associated improvement of the patient's immune function.

Overall current therapy for HIV-associated KS is still suboptimal. However with the discovery of HHV8 and the development of potent anti-HIV therapies progress is being made in patient management. Currently, except for the maintenance of immune system function, there is no effective prophylactic treatment for HIV-associated KS, therefore novel therapies such as thalidomide warrant further investigation as they may be important in the prevention and / or treatment of KS, especially in its early stages.

4. b. The significance of herpesvirus infections in the immunocompromised host

In all types of immunocompromised patient herpesvirus infections and disease cause a broad spectrum of morbidity and significant mortality. As all members of the family can become latent following primary infection there is a constant threat of viral reactivation in individuals with a reduced level of immune surveillance. In HIV-infected patients most herpesviral disease occurs when the CD4 count has dropped below 150 (Pertel *et al.,* 1992), in patients receiving immunosuppressive therapy following organ transplantation it is likely to occur in the first 100 days post-transplant, before restoration of host immunity (Peters *et al.,* 1991b and Meyers *et al.,* 1980).

Of all the herpesvirus disease occurring in these immunocompromised patients CMV infection is the most common and is of the greatest clinical significance. In the study of herpesvirus genome prevalence in post-mortem samples (section 3. e.) 19 of 31 patients (61%) had clinical evidence of CMV disease and 19 of 31 also had CMV DNA detected in either their eye or CNS. In the study of CMV in transplant recipients (section 3. d.) 14 of the 19 patients (74%) known to have had previous CMV infection had systemic CMV DNA detected at least once in the post-transplant period.

If untreated CMV causes a wide spectrum of disease in these immunocompromised patients. HIV-infected patients are at risk of CMV-induced cytocidal destruction of their retinal cells, which may rapidly result in patient blindness. CMV can also infect and destroy neuronal cells causing encephalitis and polyradiculopathy (reviewed by Bloom and Palastine, 1988). It is also likely that CMV and HIV interact at the molecular level to up-regulate their respective rates of replication, thereby accelerating host immune destruction (Duclos *et al.*, 1989 and Wiley *et al.*, 1988). In bone marrow transplant recipients the predominant site of CMV disease is the lungs where tissue damage is caused not by the viral lysis of cells, but by an immuno-pathological process involving the incorrect activation of cytotoxic T-lymphocytes (Shanley *et al.*, 1987).

In contrast to most other viral infections two therapeutic agents are available for the treatment of CMV disease. Both GCV and PFA are highly active against CMV infection (reviewed by Serody, 1993) and may arrest disease progression in the eye, brain and lungs of immunocompromised patients. However what appears crucial to the effective use of these treatments is their early instigation, preferably prior to the presentation of clinical symptoms (Eddleston *et al.*, 1997 and Reed *et al.*, 1988). As shown in section 3. d. it is possible with appropriate prophylaxis and the early detection of CMV replication, by PCR analysis, to eliminate CMV disease from a cohort of acutely immunosuppressed transplant patients. In the absence of prophylaxis and treatment approximately 60% of the CMV-infected patients in this study would have been expected to develop CMV disease, with up to 10% dying from the disease by day 100 post-transplant (Reusser *et al.*, 1990). Unfortunately it was not possible to conduct a parallel study of CMV surveillance in a cohort HIV-infected patients, however other groups have reported similar benefits to those described, in section 3. d. for the bone marrow transplant patients, with the use of CMV-prophylaxis regimens in HIV-infected groups (Katlama, 1996 and Danner, 1995).

Of the other herpesviruses investigated in this thesis only EBV was associated with specific disease. The study of post-mortem material (section 3. e.) extended previous reports of EBV-associated morbidity in HIV-infected patients (Pednaultt *et al.*, 1992 and MacMahon *et al.*, 1991), with 3 of 4 patients with CNS lymphoma having EBV DNA detectable in their CNS. Systemic EBV DNA was also detected in 60% of HIV-infected patients analysed in the study of HHV8 DNA prevalence (section 3. a. 4) confirming its high prevalence in this patient group. HSV-1 and HSV-2 were rarely detected in the study of post-mortem material, HHV6 and HHV7 were not detected even though they have an almost 100% seroprevalence in most populations. Although VZV DNA was detected in 4 patients, in none was it associated with disease.

Without methods for eradicating herpesvirus infections after the establishment of latency there is little chance of reducing the number of immunosuppressed patients at risk of developing CMV, EBV and HHV8-associated disease during their period of immune-suppression. Surveillance programs for the rapid detection of emerging infection may be useful in allowing early treatment, however the only truly effective mechanism of combating herpesviral infections, and preventing associated disease, is a return of the patient to a state of restored immunological control. In transplant patients this is possible after reconstitution of the received bone marrow. For HIV-infected patients it would appear that the new generation of protease inhibitor drugs (discussed in section 4. a.) are currently the most effective means of restoring immune function. Only by allowing the patient some form of immune regeneration will effective long-term management of herpesvirus infection be possible.

4. c. Increasing the efficiency of molecular diagnostics for herpesvirus infections

The use of molecular techniques, such as PCR, for the detection of viral genomes has significantly increased the efficiency of herpesviral diagnosis (Storch *et al.*, 1994, Miller *et al.*, 1994, and reviewed by Griffiths, 1993) and as the study of CMV infection in transplant recipients (section 3. d.) showed, a reliance on tissue culture and fluorescent detection assays, such as the DEAFF test, must now be considered inappropriate for the early identification of herpesviral infection. The speed of viral detection has also been improved, with most PCR assays providing "same-day" results. However, although molecular techniques have provided a significant improvement in viral diagnosis there is an increased financial and technical cost in providing such a service. Investigation of herpesviral infections has, until recently, been achieved by the individual analysis of each genome species separately (Mitchell *et al.*, 1994). As detailed in section 3. f. this method is extremely costly and labour intensive, and has led to the investigation of alternative methods, such as the multiplex PCR assay. The assay described in section 3. f. allows for the detection of up to six different herpesviruses, simultaneously, at the same level of detection sensitivity as for the individual nested PCR's previously used. Although designed for the detection of herpesvirus genomes the same format should be appropriate for the analysis of any variety of infectious pathogens, depending on patient type and clinical need.

As well as its use as a diagnostic assay the multiplex system is also of value as an epidemiological research tool, reducing the number of assays required to determine the prevalence of target sequences in a clinical sample panel. Indeed the analysis of herpesviral infections in the eyes and CNS of HIV-infected patients (section 3. f.) would have been completed at a greatly reduced cost, and in far less time, had the multiplex assay then been available.

In addition to assay formats such as the multiplex system it will be important, for the future management of herpesvirus infections, to develop techniques such as quantitative PCR and reverse transcriptase PCR. Quantitative PCR has already been shown to be a valuable aid in deciding on treatment strategy in transplant patients with CMV infection (Peiris *et al.*, 1995 and Ljungman *et al.*, 1996) and as mentioned in section 3. d. an ability to monitor of the levels of CMV DNA in such patients may allow a more selective approach to the use of PFA and GCV therapy. Already in this thesis the quantification of systemic HHV8 DNA has been found, in section 3. b., to mirror the response of clinical KS to a variety of therapeutic agents. Reverse transcriptase PCR enables the detection of viral messenger RNA and hence actively replicating virus. This technique has been found valuable in predicting herpesvirus emergence from latency, especially of CMV, and may be an early indicator of subsequent disease in immuno-compromised patient populations (Randhawa *et al.*, 1994 and Gozlan *et al.*, 1996).

4. d. Conclusions

Of all the viral infections affecting the immunocompromised host those caused by the family *herpesviridae* are the most widespread and clinically important, making the understanding of the epidemiology of these pathogens vital for effective patient management. In this thesis I have described the prevalence of all eight human herpesviruses in a variety of patient populations, and suggested improved diagnostic assay techniques for determining the presence of individual herpesvirus infections. Also investigated was the association between the most recently described human herpesvirus, HHV8, and Kaposi's sarcoma. The studies described support the argument that HHV8 is causal of KS, is sexually transmitted, and that its detection is of significant clinical value in the HIV-infected patient population.

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Appendix A: Culture media and buffer recipies

All media and buffers were made up in sterile double-distilled water unless otherwise stated.

Tissue Culture Media

Complete RPMI

2.0 X 10^{-3} M L-glutamine
100 units / ml penicillin
100 µg / ml streptomycin
2.5 µg / ml fungazone
10% (v/v) FCS
made up in 1 X RPMI
(Dutch modification)

Freezing medium

10% (v/v) DMSO
30% (v/v) FCS
Made up in 1 X RPMI
(Dutch modication).

HEL growth medium

10% (v/v) FCS
100 units / ml penicillin
100 µg / ml streptomycin
2.5 µg / ml fungazone
made up in 1x minimal Eagles medium

Hel maintenance medium

2% FCS (v/v)

100 units / ml penicillin

100 µg / ml streptomycin

2.5 µg / ml fungazone

made up in 1x minimal Eagles medium

Throat swab medium (virus transport medium)

5% FCS (v/v)

100 units / ml penicillin

100 µg / ml streptomycin

2.5 µg / ml fungazone

made up in 1x Earle's salts

Buffers for serological and ELONA studies

Phosphate buffered saline (PBS)

1.5 X 10^{-1} M NaCl

1.5 X 10^{-3} M KH_2PO_4

6.3 X 10^{-3} M $Na_2HPO_4.12H_2O$

2.6 X 10^{-3} M KCL

pH 7.2

20X SSC

3.0 M NaCl

3.0 X 10^{-1} M sodium citrate

pH 7.0

HHV8 IIF wash and diluent buffer

3% (v/v) FBS

from 20% stock

Diluted in PBS.

Carbonate / Bicarbonate diluent

0.05M carbonate / bicarbonate

pH 9.6

HHV8 ELISA Blocking buffer

5% (w/v) dried skimmed milk

0.1% (v/v) Tween 20

Diluted in PBS.

HHV8 ELISA coating buffer

HHV8 orf 65.2

Diluted in 0.1 M $NaHCO_3$

HHV8 ELISA substrate buffer

1 mg / ml dinitrophenyl phosphate

Diluted in 1M diethanolamine

ELONA coating buffer

5 mg / ml (w/v) streptavidin

Diluted in 0.1 M 0.1 M $NaHCO_3$

ELONA blocking buffer

0.2% FBS (v/v)

(from 20% stock)

Diluted in PBS.

ELONA sample diluent

0.1% (v/v) FBS

from 20X stock

0.1% (v/v) Tween-20

0.01% (w/v) sodium azide

Diluted in PBS

ELONA wash buffer (TTA)

10 mM Tris-HCl (pH 7.5)

0.05% (v/v) Tween-20

0.1% (w/v) sodium azide

Diluted in PBS

ELONA probe diluent

0.1% FCS (v/v)

from 20% stock

0.1% (v/v) Tween-20

Diluted in 10X SSC

(from 20X stock)

ELONA probe wash buffer

0.05% (v/v) Tween-20

Diluted in PBS.

Mounting Solution

90% (v/v) glycerol solution
Diluted in PBS.

Buffers for molecular studies

PCR reaction buffer (10 X RB)

5.0×10^{-1} M KCl

2.0×10^{-1} M Tris

1.5×10^{-2} M $MgCl_2.6H_2O$

0.1% (w/v) gelatin

pH 8.4

Autoclave KCl / Tris and adjust pH before addition of autoclaved $MgCl_2.6H_2O$ / gelatin

Gel loading buffer

30% (v/v) glycerol

0.25% (w/v) xylene cyanol

TAE (50x)

1.0 M Tris

6% acetic acid

5.0×10^{-2} M EDTA

(from pH 8 stock)

Agarose gel

1 X TAE (from 50X stock)

2% (w/v) agarose

0.05% (w/v) etidium bromide

Glycigel freezing medium (for 1 litre)

375 ml glycerol

1.8g EDTA

15g gelatin

5.4g NaCl

1.0g sodium azide

make up to 1 litre with distilled water

heat to 70°C until all gelatin dissolved, cool and aliquot

Glycigel lysis buffer

1 X 10^{-2} M Tris-HCl

(pH 7.5)

1% (v/v) Triton X100

3.2 X 10^{-1} M sucrose

5.0 X 10^{-2} M $MgCl_2$

Sterilise Tris-HCl / sucrose / $MgCl_2$ and adjust pH before addition of Triton

Glycigel extraction buffer

1 X 10^{-2} M Tris-HCl

(pH 8.3)

5.0 X 10^{-2} M KCl

2.5 X 10^{-3} M $MgCl_2$

0.0001% (w/v) gelatin

0.45% (v/v) Tween 20

0.45% (v/v) Nonidet P40

Sterilise Tris / EDTA / NaCl by autoclaving and adjust pH before addition of gelatin / Tween-20 / Nonidet P40

SDS-extraction buffer

1.0×10^{-2} M Tris

2.5×10^{-2} M EDTA

1.0×10^{-1} M NaCl

0.5% (w/v) SDS

100 µg / ml protinase K

pH 8.0

Sterilse Tris / EDTA / NaCl by autoclaving and adjust pH before addition of SDS

Add protinase K just prior to use.

TEN buffer

1.0×10^{-2} M Tris

1.0×10^{-3} M EDTA

5.0×10^{-2} M NaCl

Buffer saturated phenol

40% (v/v) phenol

12% (v/v) TEN buffer

0.04% (w/v) hydroxyquinoline

TE buffer

1.0×10^{-2} M Tris

1.0×10^{-3} M EDTA

Appendix B: Publications arising from this thesis

Howard M, Brink N, Miller R and Tedder R.
Association of human herpes virus with pulmonary Kaposi's sarcoma. *Lancet.* (1995) **346:** 712.

Whitby D, **Howard MR**, Tenant-Flowers M, Brink NS, Copas C, Boshoff C, Hatzioannou A, Suggett FEA, Aldam DM, Denton AS, Miller RF, Weller IVD, Weiss RA, Tedder RS and Schulz TF.
Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet.* (1995) **346:** 799-802.

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Kaposi's sarcoma and KSHV. *Lancet.* (1995) **346:** 1359-60.

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Regression of AIDS-related Kaposi's sarcoma during therapy with thalidomide. *Clin Infec Dis.* (1996) **23:** 501-03.

Simpson GR, Schulz TF, Whitby D, Cook PM, Boschoff, Rainbow L, **Howard MR**, Gao SJ, Bohenzky RA, Simmonds P, Lee C, de Ruiter A, Hatzakis A, Tedder RS, Weller IVD, Weiss RA and Moore PS.
Prevalence of HHV-8 infection as measured by antibodies to a recombinant capsid protein and a latent immunoflouresence antigen. *Lancet.* (1996) **348:** 1133-1138.

Howard MR, Whitby D, Bahadur G, Suggett F, Boshoff C, Tenant-Flowers, Schulz TF, Kirk S, Matthews S, Weller IVD, Tedder RS and Weiss RA.
Detection of human herpesvirus 8 DNA in semen from HIV-infected individuals but not healthy semen donors. *AIDS.* (1997) **11:** F15-19.

Howard MR, Brink NS, Tedder RS and Miller RF. Detection of Human Herpesvirus 8 in the bronchoalveolar lavage fluid from HIV-infected patients with and without pulmonary Kaposi's sarcoma. *J AIDS and Hum Retrovir.* (1997) **14:** A20.

Bower M, **Howard MR**, Tedder RS, Gracie F, Philips RH and Fife K.
A phase II study of thalidomide for Kaposi's sarcoma: activity and correlation with human herpesvirus 8 DNA titre. *J AIDS and Hum Retrovir.* (1997) **14:** A45

P. Monini, **M.R. Howard**, Rimessi P., de Lellis L., Schulz T.F. and Cassai E. Human herpesvirus DNA in prostate and semen from HIV-negative individuals in Italy. *AIDS.* (1997) **11:** 1530-1532.

Howard MR and Bahadur G.
Kaposi's sarcoma-associated herpesvirus (KSHV)- a concern for human reproduction. *Hum Rep.* (1997) **12:** 2567-2571.

Howard MR, Brink NS, Whitby D, Tedder RS and Miller RF.
Association of Kaposi's sarcoma-associated herpesvirus (KSHV) DNA in bronchoalveolar lavage fluid of HIV-infected persons with bronchoscopically diagnosed tracheobronchial Kaposi's sarcoma. *Sex Transm Dis.* (1998) **74:** 27-31.

In Press:

Brink N.S., Sharvell Y., **M.R. Howard**, J.D. Fox, M.J.G. Harrison and R.S. Miller.
Detection of Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus DNA in cerebrospinal fluid from HIV-infected persons with neurological disease. *J. Neurol and Neurosurg.* (in press).

Fife KM, **Howard MR**, Tedder RS, Gracie F, Philips RH andBower M.
A phase II study of thalidomide for Kaposi's sarcoma: activity and correlation with human herpesvirus 8 DNA titre. *Int J Sex Trans Dis.* (in press)

Submitted:

S. Kirk, R.S. Tedder and **Howard M.R.** A nested PCR multiplex reaction allowing the single copy detection and differentiation of six clinically important herpesvirus genomes.(submitted *J. Clin. Micro.*)

Appendix C: Post-mortem ocular and neurological herpesvirus PCR results

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 1	Frontal lobe:	-	-	-	+	-	-	-	+
Right eye	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Left eye	Vitreous:	-	+	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Patient 2	Frontal lobe:	-	-	-	-	-	-	-	-
	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Patient 3	Frontal lobe:	-	-	-	+	-	-	-	ns
	Vitreous:	-	-	-	-	-	-	-	ns
	Aqueous:	-	-	-	-	-	-	-	ns
Patient 4	Frontal lobe:	-	-	-	+	-	-	-	-
	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Patient 5	Frontal lobe:	-	-	-	-	-	-	-	-
	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 6	Frontal lobe:	-	-	-	+	-	-	-	-
	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Patient 7	Frontal lobe:	-	-	-	+	-	-	-	-
	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Patient 8	Frontal lobe:	-	-	-	-	-	-	-	+
	Vitreous:	-	-	-	-	-	-	-	+
	Aqueous:	-	-	-	-	-	-	-	+
Patient 9	Frontal lobe:	-	-	-	+	-	-	-	-
	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Patient 10	Frontal lobe:	-	-	-	-	-	+	-	-
	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Patient 11	Frontal lobe:	-	-	-	-	-	-	-	-
	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 12	Frontal lobe:	-	-	-	-	-	+	-	-
	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	+	-	-
Patient 13	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Left eye	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	-	-
Patient 14	CSF:	-	-	+	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Left eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	+
Patient 15	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Left eye	Vitreous:	-	-	-	+	-	-	-	-
	Aqueous:	-	-	-	+	-	-	- -	

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 16	CSF:	-	-	-	-	NT	+	NT	-
Right eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Left eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Patient 17	CSF:	-	-	-	-	NT	-	NT	-
Right eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Left eye	Vitreous:	+	-	-	-	-	-	--	
	Aqueous:	-	-	-	-	-	-	-	-
Patient 18	CSF:	-	-	-	-	NT	-	NT	-
Right eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Left eye	Vitreous:	-	-	-	-	-	-	-	-
	Aqueous:	-	-	-	-	-	-	-	-
Patient 19	CSF:	-	-	-	-	NT	+	NT	-
Right eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-
Left eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 20	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Patient 21	CSF:	-	-	-	+	NT	+	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Patient 22	CSF:	-	-	-	-	NT	+	NT	-
Right eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-
Patient 23	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 24	CSF:	-	-	+	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Patient 25	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Patient 26	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-
Patient 27	CSF:	-	-	+	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-

		HSV-1	HSV-2	VZV	CMV	HHV-6	EBV	HHV-7	HHV-8
Patient 28	no CSF								
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Patient 29	CSF:	-	-	-	+	NT	-	NT	-
Right eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Left eye	Vitreous:	-	-	-	+	NT	-	NT	-
	Aqueous:	-	-	-	+	NT	-	NT	-
Patient 30	CSF:	-	-	+	-	NT	-	NT	-
Right eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-
Left eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	+	-	NT	-	NT	-
Patient 31	CSF:	-	-	-	-	NT	-	NT	-
Right	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-
Left eye	Vitreous:	-	-	-	-	NT	-	NT	-
	Aqueous:	-	-	-	-	NT	-	NT	-

NS = no sample
NT = not tested

A nested PCR multiplex reaction allowing single copy detection and differentiation of six clinically important herpesvirus genomes.

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Running title: Multiple herpesvirus genome detection by PCR

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Abstract

Rapid and accurate detection of herpesvirus infections in the immunocompromised individual is vital for effective patient management. Recently the assay of choice for such analysis has been the polymerase chain reaction, however large-scale use of this assay is expensive and time-consuming. To overcome the problems inherent in testing clinical samples for a number of herpesvirus genomes by individual nested PCR amplification, we have developed a multiplex PCR assay with an enzyme-linked oligonucleotide detection system. This assay allows simultaneous detection of HSV-1, HSV-2, VZV, EBV, CMV and KSHV DNA. The assay has the same detection sensitivity, of a theoretical single target genome, as our established nPCR's reactions and shows a 95% concordance in the detection of herpesvirus genomes from a large panel of clinical specimens. Use of the mPCR-ELONA provides as complete a herpesvirus genome detection service as our existing individual nested PCR reactions with a saving of up to 70% in cost and time taken to provide a sample result. Such an assay has an important future role in the clinical support we provide to our patients at increased risk of serious herpesvirus infections.

Introduction

Currently eight human herpesviruses have been described. These are herpes simplex type 1 (HSV-1) and type 2 (HSV-2) viruses, varicella-zoster virus, (VZV), Epstein Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6) and type 7 (HHV-7) and Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus type 8 (HHV-8). Although all these viruses, with the exception of HHV-7, have been associated with specific diseases, the symptomology of herpesvirus infections, particularly in the immunocompromised, can manifest themselves as non-specific syndromes. The severity of a number of these diseases, especially in the immunocompromised host, together with the development of a range of anti-herpesvirus chemotheraputic agents has made the rapid and accurate diagnosis of herpesvirus infections increasingly important in long-term patient management.

At present there are a variety of methods available for the determination of herpesvirus involvement in the clinical setting. Classically these were based upon the relative ease with which certain members of the family, such as HSV-1, HSV-2 and CMV, could be cultured *in vitro* directly from patient material (9). However with the transient expression cultures such as DEAFF, viral antigenaemia detection and the development of molecular diagnostic techniques such as the polymerase chain reaction (PCR), it is now possible to detect and measure extremely low levels of herpesvirus. Such analysis has been of particular advantage in identifying herpesvirus involvement in neurological and ocular diseases (1,16,21,23). The ultra-sensitive assay currently in

use in the Department of Virology, University College London School of Medicine (UCLMS) is the nested PCR (nPCR) with individual reactions available for detection of each of the most clinically significant herpesviruses; HSV-1, HSV-2, VZV, CMV, EBV and KSHV (10,16).

Following an audit of a three month period it was found that of 352 samples received for herpesvirus analysis, only 75 contained any detectable herpesvirus DNA. However to provide the clinically-requested herpesvirus "screen" for this number of samples, nearly 1200 individual amplification reactions, together with a similar number of control reactions, were required. As herpesviral DNA was detected in less than 10% of the assay reactions it was felt a different system was required to exclude rapidly any negative samples, while reliably identifying those which were positive for further analysis and confirmation.

The approach chosen has been the development of a herpesvirus multiplex nested PCR (mPCR) in which the primers for amplification of HSV-1, HSV-2, VZV, EBV, CMV and KSHV DNA are present within a single reaction mix. This means that if target genome from any of these viruses is present in a clinical sample it will be amplified. To visualise the products of the multiplex reaction an enzyme-linked oligonuclotide assay (ELONA) was constructed based upon the principle developed by Whitby and Garson (25) for the quantification of hepatitis C viraemia. This system allows rapid, high-throughput analysis of the mPCR amplification products

and relies upon robust technology utilised in enzyme-linked immunoassays for over 20 years.

Following development of the multiplex assay and ELONA detection system, 192 clinical samples were analysed by both the existing individual nPCR's, visualised by conventional gel electrophoresis, and the mPCR-ELONA to determine the level of concordance between the two systems. The sensitivity of the mPCR-ELONA was also determined by Poisson analysis using analytes of known genomic content.

Materials and Methods

Sample processing.

Samples (table 1) for herpesviral DNA analysis were stored at 4°C until processing. The samples were centrifuged at 15,000rpm for 5 minutes to remove cellular material before heating the supernatant at 96°C for 10 minutes and directly adding the boilate to the PCR. DNA from EDTA blood specimens was purified using a commercial extraction kit (QiaAmp Blood Kit, Qiagen Ltd), and DNA from glycigel-preserved blood and tissue biopsies was prepared as previously described (22,24).

PCR reaction conditions.

The primers used in the first and second rounds of the individual nPCR's for the detection of HSV-1, HSV-2, VZV, CMV, EBV and KSHV DNA (table 2) were used in the mPCR first and second round amplifications. The individual primer sets and reaction conditions for the detection of CMV, HSV-2, EBV and KSHV have previously been shown capable of single copy genome detection (10, 16). The primer sets and reaction conditions for HSV-1 and VZV are able to detect 0.01 infectious units and 0.01 infected cells respectively (16).

For the optimisation of the mPCR a range of cycling parameters, primer concentrations and enzymes were investigated. Additionally the second round

(nested) sense primers for each virus were modified by 5'-end labelling with biotin. The cycling conditions were investigated as follows: The first round amplification was varied between 30 and 40 cycles, while in the second round the cycle number was varied between 20 and 35; the denaturation temperature was either 94°C or 95°C for 10 seconds to 60 seconds; the annealing temperature ranged between 46°C and 50°C for 10 seconds to 60 seconds. Primer concentrations in the second round were varied between 25ng and 70ng per reaction. *Taq* (Perkin Elmer) and *Taq*gold (Perkin Elmer) DNA polymerases were compared with the latter requiring a 95°C, 12 minute activation step prior to the commencement of thermal cycling. The reaction buffer used throughout was 200µM of each dNTP, 10mM Tris-HCl (pH 8.3), 50mM KCl and 1.5mM $MgCl_2$.

Multiplex PCR product detection

An enzyme linked oligonucleotide assay (ELONA) based on that developed by Whitby and Garson (25) was modified to a colorimetric assay (figure 1). Clear 96-well microtitre plates (Nunc, Maxisob) were coated with 5µg/ml streptavidin (Sigma) in carbonate / bicarbonate coating buffer (pH 9.6) overnight at 20°C. The wells were blocked using 0.2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Various volumes of mPCR products, ranging from 2µl to 20µl were added to the microtitre plate in a sample diluent of 0.1% BSA, 0.1% Tween-20 and 0.01% sodium azide in PBS and incubated at 45°C for times ranging between 30 minutes and 75 minutes. This allowed the capture of the biotinylated PCR products onto the solid

phase. The plate was then washed using 10mM Tris-HCl (pH7.5), 0.05% Tween-20, 0.1% sodium azide (TTA) wash buffer before treatment with 0.15M NaOH for 2 to 5 minutes. at 20°C to produce single stranded amplicons before repeat washing.

Anti-sense oligonucleotide probes were designed against internal sequences of each of the 6 target virus PCR products (table 3). These probes were modified to be either 5'-end labelled with biotin or horse radish peroxidase. Various concentrations of the probes, diluted in 10x saline-sodium citrate buffer (SSC), 0.1% BSA and 0.1% Tween-20 probe diluent, were incubated on the plate for 20 to 40 minutes before being washed in PBS / 0.05% Tween-20. The biotinylated probes had an additional incubation with a streptavidin-horse radish peroxidase conjugate (Dako) before the addition of the substrate, 3,3',5,5'-tetramethylbenzidine (TMB) in tri-sodium citrate and hydrogen peroxide (Murex diagnostics). Plates were then incubated at 37°C for 15 to 40 minutes to allow colour development before stopping the reaction with 2N H_2SO_4. The optical density of the reaction was then measured at 450nm, and normalised by dividing the test optical density (OD) by the negative OD values.

Initial screening of mPCR products was performed using a mixed pool of all 6 oligonucleotide probes. Any positive samples were re-probed, using the remaining amplification product, with each probe individually in order to define virus type/s present.

Controls.

Positive controls for HSV-1, HSV-2 and VZV DNA detection were tissue culture fluids derived from confirmed virus isolation positive samples. CMV DNA was derived from the tissue culture adapted AD169 strain. EBV and KSHV DNA was extracted and purified from the P3HR1 and BCP-1 cell lines respectively (3). Each control was known to contain a single type of herpesvirus genome and was analysed in each mPCR-ELONA assay at approximately 10 input copies, 1 copy and 0.1 copy concentrations to determine inter-assay sensitivity.

Clinical samples (table 1).

All samples used in the multiplex PCR were samples received by the Department of Virology, UCLMS for routine herpesvirus PCR and were tested either prospectively or concurrently with the standard individual nPCR assays. Samples were said to have detectable herpesvirus genome if amplification occurred in at least 2 separate nPCR's.

Results

Assay optimisation.

As described, a wide range of PCR conditions were investigated in order to maximise the sensitivity of the mPCR assay. Optimum results were obtained using a nested PCR format with increased cycle numbers in both the first and second rounds when compared with the individual nPCRs. It was found that *Taq*gold gave lower levels of non-specific products but increased levels of specific products compared with *Taq* when the PCR products were analysed on a 2% agarose gel. This meant that an initial activation step of 95°C for 12 minutes was required in both rounds of the mPCR. The optimum cycle conditions for the first round were; 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds for 40 cycles followed by a final 7 minute, 72°C extension cycle. 100ng of the first round primers for each of the 6 target herpesviruses was included in the 50µl reaction volume with 1.25 units of *Taq*gold.

The optimum cycle conditions for the second round, following the activation step were; 32 cycles of 95°C for 30 seconds, 46°C for 30 seconds and 72°C for 30 seconds followed by 7 minute extension cycle at 72°C. The primers were used at 30ng each in a 50µl reaction volume and 1µl of first round product was transferred into the second round reaction mix. Again 1.25 units of *Taq*gold were added to each reaction tube.

For the detection of the amplified products by ELONA it was found that 5µl of the second round mPCR product, added to the streptavidin-coated microtitre plates, gave the optimum results in combination with the horse-radish peroxidase conjugated oligonucleotide probes (figure 1). The working concentration of each probe was determined empirically for each virus amplicon. After incubation at 45°C for 30 minutes, of 5µl of the mPCR product diluted in 95µl of sample diluent, the plate was washed ten times using TTA before the addition of 100µl of 0.15M NaOH for 2 minutes at 20°C, followed by a further ten washes in TTA. For initial product screening, 100µl of a pool of all 6 herpesvirus probes, diluted to their working strength in probe diluent, were added to each well and incubated at 45°C for 30 minutes. Free unbound probes were removed by washing ten times, with 1 minute soaks between each wash, in PBS Tween, before the addition of 100µl of TMB substrate at 37°C for 20 minutes. The reaction was then stopped using 2N H_2SO_4 (figure 1).

Any sample whose amplification product gave a test OD : negative OD ratio of greater than 5 by the "all-probe" ELONA was considered to contain detectable herpesvirus genome. Positive products were then retested on a second streptavidin coated plate by adding 5µl of the remaining mPCR product into each of 6 wells and incubating as in the screening assay. The working dilution of each herpesvirus probe was then made up individually and 100µl added, one to each of the six test wells (figure 1). ELONA detection was carried out as before with any well or wells showing a test OD : negative OD ratio of greater than 5 indicating the virus type/s present within the original sample.

Results obtained with the optimised protocol.

Sensitivity and specificity.

Once the mPCR reaction conditions had been optimised, serial ten-fold dilutions of each of the positive controls for the six herpesvirus genomes of interest were tested in parallel with the individual nPCR. The absolute sensitivity of the mPCR was found to be equal to that of the individual nPCRs (see methods).

To show that the mPCR-ELONA was capable of single copy genome detection, 1µl volumes of CMV and HSV-1 DNA- containing samples, which had previously been shown by Poisson distribution to contain, 0.3 and 0.1 copies of control DNA respectively, were tested in 40 replicates by both mPCR-ELONA and individual nPCR. Both assays were found to be of comparable sensitivity and detected similar numbers of single genome copies (figure 2). This analysis confirmed that any positive product derived from a theoretical single input genome would give a test OD : negative OD ratio of greater than 5 in the mPCR-ELONA.

The specificity of each internal oligonucleotide probe was demonstrated by testing DNA positive samples of each herpesvirus with: 1) a pool of all 6 probes, 2) a pool of

the 5 non-specific probes or 3) the specific probe alone. This showed that only when the probe specific for the target genome was present was the sample shown to be positive by ELONA. No competition was observed between the probes preventing amplified product detection when all 6 probes were mixed together. No cross reactivity was found between the probes and their non-specific heterotypic viral targets.

Herpesvirus genome detection in clinical samples.(Table 4).

A total of 192 samples were tested blind by mPCR having previously been tested by the individual herpesvirus nPCR's. Of these samples 70, were known to contain at least 1 detectable type of herpesvirus genome. The mPCR-ELONA, using the 6-probe screen, detected herpesvirus genome in 66 of these 70 samples giving a sensitivity of 94% compared with the individual nPCRs. Within these 70 positive samples 81 herpesviral genomes had been detected by the individual nPCR's. 77 (95%) were correctly typed by the mPCR-ELONA (figure 3). Of the 4 samples in which herpesvirus genome was not detected by mPCR-ELONA, 3 were reanalysed by nPCR and found to have extremely low copy numbers of viral genome present (table 5). There was insufficient remaining of the other sample to retest. None of the 122 samples to have no detectable herpesviral DNA by individual nPCR were shown to be positive by mPCR-ELONA (see figure 4), all gave a test OD : negative OD well below the arbitrary five-fold cut-off.

Discussion

A number of methods have been proposed by which PCR may be utilised for the simultaneous detection of diverse herpesvirus genomes (5,18,20). These have included the development of universal primers directed against the highly-conserved viral polymerase gene (11), the use of multiple primer sets in multiplex reactions with differentiation of genome amplicons by product size (19) or with the use of restriction enzyme digestion to differentiate PCR products of similar size by their fractionation patterns (11). Each of these methods have been shown to have a number of weaknesses, including a lack of detection sensitivity and a limit to the number of virus species able to be analysed (4). This study has shown that use of the nested multiplex PCR followed by ELONA detection overcomes both of these problems. Furthermore the use of three separate hybridisation reactions (nested PCR primers followed by an internal oligonucleotide probe) increases specificity and allows the production of sufficient amplified product, by increasing the cycle number, for reliable detection at the single input genome level. This enables the assay to show a 95% correlation with routine validated single genotype specific nPCR assays. It has also proved possible to include in the mPCR-ELONA 6 sets of nested PCR primers and 6 detection probes without compromising either assay sensitivity or specificity.

As well as providing rapid detection of clinically significant herpevirus genomes the mPCR-ELONA assay also has the advantage of requiring significantly less initial clinical material for analysis. Although usually not a problem with certain samples,

such as urine and BAL fluid, it is common to receive extremely small volumes of material such as CSF, and vitreous and aqueous fluids. Extensive analysis by individual PCR may quickly exhaust such samples, while as little as 10µl is sufficient for full analysis by the mPCR-ELONA.

In our hands, using the simple DNA extraction method of boiling samples, we have found very similar results to the standard nPCR, identifying only four samples where the mPCR-ELONA failed to detect viral genome. Retesting confirmed extremely low levels of genomic sequence in the three which were still available (table 5). With the development of rapid nucleic acid extraction techniques, such as silica column DNA purification (Qiagen), it is likely that DNA from larger specimen volumes can be used in future which is likely to increase the overall sensitivity of detection. Indeed, increased DNA purity may avoid the need for a nested reaction, a strategy which is likely to increase further the utility of the mPCR-ELONA.

Of most significance in terms of use in a large diagnostic virology laboratory such as ours, is the saving in cost and overall analysis time that this multiplex assay provides in comparison with individual nPCR analysis. Use of this assay is projected to result in a reduction of between 60 - 70% in the number of PCR reactions required to provide a complete herpesviral diagnostic service. Use of the assay has also been of benefit in a number of research environments especially in the determination of herpesvirus epidemiology associated with particular diseases and / or patient groups (Howard *in prep).*

In conclusion we present a technically simple and robust assay system which, while of comparable sensitivity to established nPCR assays, allows the provision of a comprehensive rapid multiplex herpesviral genome screening service. No specialised equipment is required for utilisation of the assay over that commonly found in any diagnostic department already performing nested PCR analysis. Use of this assay should provide a valuable service in the diagnosis of herpesvirus infections and be of significant benefit in allowing rapid clinical assessment of patients at greatest risk of associated disease.

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433 **Table 1** : Breakdown of sample types tested.

434

Sample type	Total number tested	Negative by individual nPCR for any herpesvirus	Positive by individual nPCR for one or more herpesvirus
CSF	118	102	16
Tissue culture isolates	32	12	20
Vesicle fluid / swab	10	2	8
Bronchio Alveolar Lavage fluid	7	3	4
Whole blood (QIAGEN)	6	1	5
Extracted white blood cells (glycigel)	8	0	8
Mid stream urine	4	0	4
Biopsy tissue	3	3	0
Ocular:			
Vitreous fluid	2	0	2
Aqueous fluid	1	1	0
Plasma	1	0	1
Totals	192	122	70

435 **Table 2**: Primer Sequences, Anneal Temperatures and Expected Product Sizes for

436 Herpesvirus DNA by Nested PCR

437

Virus (gene)	Nucleotide location and reference [a]	Primer sequence (5'-3') and reference [b]	PCR round	Anneal temp (°C)	Product size (bp)
HSV-1 (gD)	19-43 239-218 (13)	ATC ACG GTA GCC CGG CCG TGT GAC A CAT ACC GGA ACG CAC CAC ACA A (2)	1	60	221
	51-71 188-166 (16)	CCA TAC CGA CCA CAC CGA CGA GGT AGT TGG TCG TTC GCG CTG AA (2)	2	50	138
HSV-2 (gG)	363-386 546-524 (15)	TCA GCC CAT CCT CCT TCG GCA GTA GAT CTG GTA CTC GAA TGT CTC CG (2)	1	60	183
	422-441 522-503 (16)	AGA CGT GCG GGT CGT ACA CG CGC GCG GTC CCA GAT CGG CA (2)	2	50	100
VZV (gene 29)	51067-51087 51338-51315 (8)	ACG GGT CTT GCC GGA GCT GGT AAT GCC GTG ACC ACC AAG TAT AAT (14)	1	60	272
	51091-51111 51298-51279 (16)	ACC TTA AAA CTC ACT ACC AGT CTA ATC CAA GGC GGG TGC AT (16)	2	50	208
CMV (gB)	1942-1966 2091-2067 (6)	GAG GAC AAC GAA ATC CTG TTG GGC A GTC GAC GGT GGA GAT ACT GCT GAG G (7)	1	58	150
	1967-1989 2066-2044 (16)	ACC ACC GCA CTG AGG AAT GTC AG TCA ATC ATG CGT TTG AAG AGG TA (16)	2	50	100
EBV (large internal repeat)	1347-1366 1517-1498 (12)	GAG ACC GAA GTG AAG GCC CT GGT GCC TTC TTA GGA GCT GT (16)	1	50	171
	1399-1421 1495-1476 (16)	GCC AGA GGT AAG TGG ACT TTA AT GAG GGG ACC CTG AGA CGG GT (16)	2	54	97
KSHV (minor capsid protein)	1-20 233-215 (17)	AGC CGA AAG GAT TCC ACC AT TCC GTG TTG TCT ACG TCC A (17)	1	58	233
	22-41 193-174 (17)	GTG CTC GAA TCC AAC GGA TT ATG ACA CAT TGG TGG TAT AT (17)	2	50	171

438 [a] Published sequence to which nucleotide locations refer.

439 [b] Reference is given where the primer pair was taken or adapted from published PCR

440 results.

441 **Table 3**: Nucleotide Sequences and Location of 5'-Horse Radish Peroxidase-Labelled

442 oligonucleotide Probes used for the Colorimetric Detection of Herpesvirus multiplex

443 PCR Product.

444

Virus (gene)	Probe location on genome	Probe sequence (5'-3')
HSV-1 (gD)	107-89	CTC CTC CTC GTA AAA TGG C
HSV-2 (gG)	493-474	GCA TTT ACG AGA GCG TAC
VZV (gene 29)	51154-51135	CGT GTT TGC CTC CGT GAA AG
CMV (gB)	2013-1996	GAT GAA GAT CTT GAG GCT
EBV (large internal repeat)	1454-1437	GGG TGT GGT GGA GTG TTG
KSHV (minor capsid protein)	109-92	ACA CCA ACA GCT GCT GC

445 **Table 4**: Correlation of results obtained between individual nPCR and mPCR-ELONA

446

447

	Multiplex PCR result											
	HSV-1		HSV-2		VZV		CMV		EBV		KSHV	
nPCR result	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Pos	10	2	13	0	14	0	20	0	12	2	8	0
Neg	0	126	0	127	0	81	0	85	0	72	0	50

Table 5: Four samples in which herpesvirus DNA was detected by individual nPCR but not by mPCR-ELONA.

Herpesvirus type DNA detected by nPCR	Genome load by nPCR end point titration	Test / Neg OD in mPCR
HSV-1	0.1 copies/µl	1.4
EBV	1 copy/µl	1.2
EBV	IS	1.3
HSV-1	0.1 copies/µl	1.0

IS = Insufficient sample for analysis.

Figure 1: Diagrammatic representation of the optimised protocol for the detection of captured single stranded PCR products.

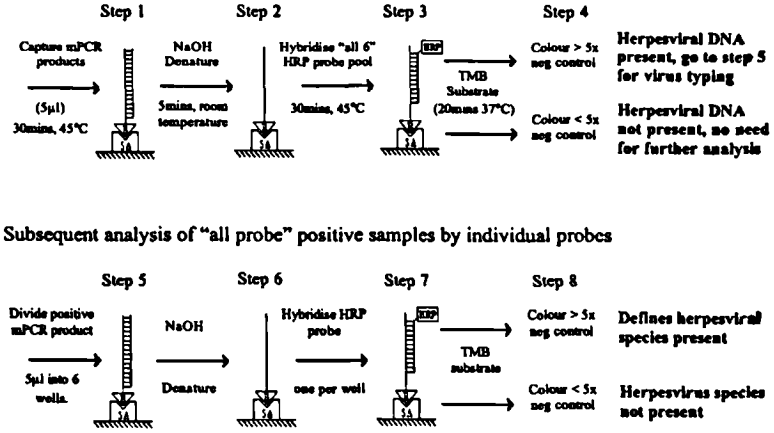
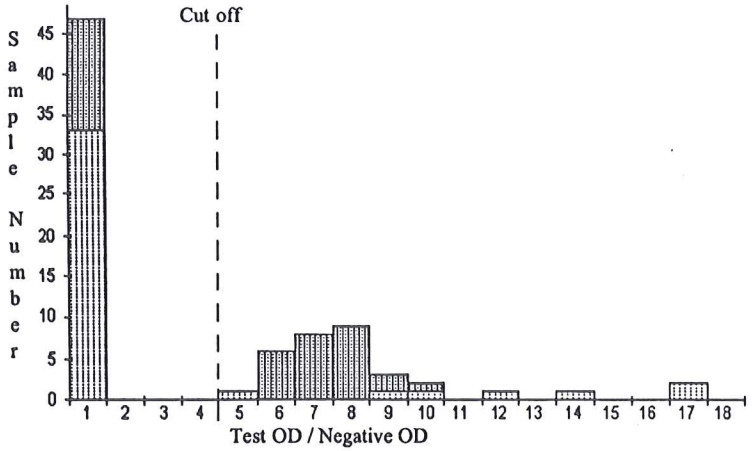


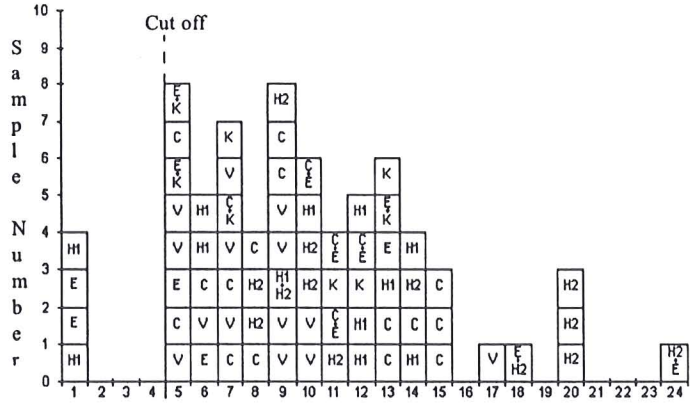
Figure 2 : Optical densities derived from mPCR-ELONA of single copy herpesviral genomes prepared by Poisson Distribution analysis.



End point dilution of CMV positive control tested 40 times by multiplex PCR. CMV genome detected in 26/40 mPCR-ELONA reactions. (Individual nPCR detected CMV genome in 14/40 reactions.)

End point dilution of HSV-1 positive control tested 40 times by multiplex PCR. HSV-1 genome detected in 7/40 mPCR-ELONA reactions. (Individual nPCR detected HSV-1 genome in 3/40 reactions.)

Figure 3 : Test / Negative optical densities obtained by mPCR-ELONA all-probe detection of 70 samples previously positive for at least 1 herpesvirus genome by individual nPCR



Uppercase letters indicate the herpesviral genomes detected in each sample by individual nPCR (H1 = HSV-1, H2 = HSV-2, V = VZV, C = CMV, E = EBV and K = KSHV).