ROLE OF THE HUMAN β-HERPESVIRUSES IN ORGAN ALLOGRAFT REJECTION FOLLOWING TRANSPLANTATION

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Abstract

In previous prospective studies conducted in our department, PCR was used to detect the three β-herpesviruses, human cytomegalovirus (HCMV), and human herpesviruses 6 and 7 (HHV-6 and HHV-7) in the blood of solid organ transplant patients. The viruses were commonly detected post-transplantation, and HCMV and HHV-6 were independently associated with graft rejection in liver recipients. HHV-7 was also associated with increased episodes of rejection in renal transplant patients. To better understand the role of these viruses in graft rejection, *in situ* techniques including *in situ* hybridisation (ISH) and immunohistochemistry (IHC) were developed to detect these viruses in renal and liver allograft biopsies from patients in the original prospective studies. HCMV DNA was detected in a significant proportion of liver and renal biopsies (approximately 50%) by ISH, with detection being widespread especially in renal allografts. The presence of HCMV DNA in the biopsies is likely to represent low level HCMV replication not detectable by IHC. HCMV was not statistically associated with either renal or liver allograft rejection by ISH. It remains possible that HCMV infection in the graft leads to dysfunction and is clinically interpreted as allograft rejection. HHV-6 and HHV-7 were not detected in any allografts by ISH or IHC.

In addition, an uncommon form of HHV-6 persistence is characterized by high viral loads in blood and integration of viral sequences into host cell chromosomes. Fluorescence *in situ* hybridisation (FISH) was developed to examine integration events in a healthy individual with a consistently high HHV-6 load and also in a case of genetic transmission of integrated virus through stem cell transplantation. In the former individual, integrated HHV-6 was identified on chromosome 11p15.5 and in the latter shown in the donor and the recipient (post-transplantation only) on chromosome 17p13.3. The confounding effect of HHV-6 integration must be considered when investigating novel disease associations of HHV-6.

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List of Abbreviations

AAV	Adeno-associated virus
ABC	avidin biotin complex
ACV	aciclovir
ADCC	antibody-dependent cell-mediated cytotoxicity
AEC	3-amino-9 ethyl carbazole
AECA	anti-endothelial cell antibody
ALG	anti-lymphocyte globulin
AP	alkaline phosphatase
APES	aminopropyl tri-ethoxysilane
APC	antigen presenting cell
ATG	anti-thymocyte globulin
Ab	antibody
Ag	antigen
BAL	bronchoalveolar lavage
BCIP	5-bromo-4-chloro-3-indoylphosphate
BMT	bone marrow transplantation
BSA	bovine serum albumin
bp	base pairs
CAN	chronic allograft nephropathy
CBMC	cord blood mononuclear cell
CDV	cidofovir
CID	cytomegalic inclusion disease
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebral spinal fluid
CTL	cytotoxic T lymphocyte
$CaCl_2$	calcium chloride
CsA	cyclosporin A
cAMP	cyclic AMP
cDNA	complementary DNA
DAB	3, 3-diaminobenzidine
DAPI	propidium iodide and diamino-2-phenylindole
DCT	distal convoluted tubules

DEAFF	detection of early antigen foci
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
D/R	donor/recipient
DSA	donor specific antibodies
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
DNase	deoxyribonuclease
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
E	early
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ES	exanthem subitum
E. coli	*Escherichia coli*
FCS	foetal calf serum
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocynate
FasL	Fas ligand
GA	golgi apparatus
GPCR	G-protein coupled receptor
GVHD	graft versus host disease
gp	glycoprotein
H and E	hematoxylin and eosin
HEL	human embryonic lung
HBLV	human B-lymphotropic virus
HCl	hydrochloric acid
HCMV	human cytomegalovirus

HBV	hepatitis B virus
HCV	hepatitis C virus
HHV	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSV	Herpes simplex virus
H_20	water
hr	hour
ICTV	International Committee on the Taxonomy of Viruses
IHC	immunohistochemistry
IE	immediate early
IFN	interferon
IL	interleukin
IPTG	isopropyl β-D-thiogalactopyranoside
ISH	*in situ* hybridisation
Ig	immunoglobulin
iv	intravenous
KIR	killer inhibitory receptor
kbp	kilobase pair
kDa	kilodalton
L	late
LAT	latency associated transcripts
LB	luria broth
LCL	lymphoblastoid cell line
LFA	leukocyte function antigen
LiCl	lithium chloride
MDBP	major DNA binding protein
MEM	minimum essential medium
MCMV	mouse cytomegalovirus
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
MIEP	major immediate early promoter
MIP	macrophage inflammatory protein

MMF	mycophenolate mofetil
MOPS	morpholinopropanesulphonic acid
MS	multiple sclerosis
MTP	major tegument protein
MAb	monoclonal antibody
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
mg	milligram
min	minute
ml	millilitre
mM	millimolar
NASBA	nucleic acid sequence-based amplification
NBT	nitroblue tetrazolium
NFAT	nuclear factor of activated T-cells
NK	natural killer
NaCl	sodium chloride
ng	nanogram
nm	nanometer
ORF	open reading frame
OBP	origin binding protein
ori	origin of DNA replication
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCT	proximal convoluted tubules
PDGF	platelet-derived growth factor
PFA	phosphono formic acid
PFGE	pulsed field gel electrophoresis
PHA	phytohaemagglutinin
PR	parvovirus rep gene homologue
PTC	peritubular capillaries
PTLD	post-transplantation lymphoproliferative disease
Pen	penicillin

pp	phosphoprotein
QC-PCR	quantitative competitive polymerase chain reaction
RANTES	regulated upon activation, normal T expressed and secreted
RCMV	rat cytomegalovirus
RNA	ribonucleic acid
RNase	ribonuclease
RSV	respiratory synctial virus
RT	reverse transcriptase
SOT	solid organ transplantation
SMC	smooth muscle cell
SSC	saline-sodium citrate
sec	seconds
Strep	Streptomycin
T	thymidine
TAP	transporter associated with Ag processing
TBE	90 mM Tris-borate, 2 mM EDTA
TCR	T cell receptor
TIF	transcription-inducing factor
TNF	tumor necrosis factor
TVS	transplant vascular sclerosis
Taq	*thermus aquaticus* DNA polymerase
temp	temperature
tRNA	transfer ribonucleic acid
UDG	uracil-DNA glycosylase
UV	ultraviolet light
µg	microgram
µl	microlitre
µM	micromolar
µm	microns
V	volts
VACV	valaciclovir
VAP	vascular adhesion protein
VBDS	vanishing bile duct syndrome
VCAM	vascular cell adhesion molecule

VGCV	valgancyclovir
VLA	very late antigen
VZV	varicella zoster virus
vICA	viral inhibitor of caspase activation
vMIA	viral mitochondria-localized inhibitor of apoptosis
w/v	weight-to-volume ratio
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter one - General Introduction

1.1 Transplantation

Transplantation is used to describe the surgical procedure by which healthy organs, tissues or cells (graft) are transferred from one individual (the donor) to another in need of the transplant (the recipient). Over the years, advances in surgical techniques, tissue typing, immunosuppressive therapy, and the management of infection post-transplantation has led to substantial improvement in the quality of life and survival rates following solid organ transplantation (SOT) (reviewed in Patel and Paya, 1997). SOT has now become a therapeutic option for many end-stage diseases e.g. alcoholic cirrhosis, insulin-dependent diabetes mellitus, and chronic glomerulonephritis. However, infection and treatment are closely interrelated, and with the advent of newer and more potent immunosuppressive regimens aimed at reducing allograft rejection, an increase risk and morbidity of infectious complications in transplant patients have resulted (Villacian and Paya, 1999). This thesis examines the potential pathogenic role of the human β-herpesviruses in the post-transplantation setting and in particular focuses on the role of these viruses in organ allograft rejection.

1.1.1 Immunological basis of allograft rejection

Allografts are grafts donated from one person to a genetically different individual of the same species. In humans the majority of organ grafts from one individual to another are *allografts*, unless an identical twin is available as a donor. Allografts express alloantigens which are recognized as foreign by the recipient and therefore result in a rejection response. Allograft rejection is a major problem in SOT as it can lead to either acute or chronic loss of the graft. The primary immunological event responsible for initiating a complex network of cellular and humoral interactions that ultimately leads to graft rejection is the recognition of non-self antigens (Ags) by the recipient's T lymphocytes (allorecognition). The second part involves activation, proliferation and differentiation of allospecific T lymphocytes into effector cells or to provide help to other cells to inflict damage to the graft.

1.1.1.1 Allorecognition

It is generally agreed that there are two nonmutually exclusive pathways of allorecognition, the direct and the indirect pathways (reviewed in Game and Lechler, 2000). Allorecognition was traditionally considered to occur by the direct pathway, where the recipient's T lymphocytes directly recognize intact allogeneic human leukocyte antigens (HLA) (usually class I) expressed on the surface of donor antigen presenting cells (APCs). However, in the early 1980s Lechler and Batchelor proposed an alternative mechanism, namely the indirect pathway of allorecognition, whereby donor HLA alloantigens are shed by donor cells, taken up and processed by recipient APCs and presented to the recipients T lymphocytes in the groove of self-HLA (usually class II).

1.1.1.2 T lymphocyte activation and response

The immune response to alloantigen can be described as a sequence of three signals (reviewed by Marion, 2003). The first signal involves the specific interaction of T cell receptors (TCR) with allopeptides bound to the groove of self or allo-HLA molecule. This interaction between TCR and allopeptides has high specificity but low affinity and requires a second signal (also known as "costimulation") before T-cell activation can occur. This second signal is provided by ligands on the APC. Although many costimulatory molecules have been identified, the CD28 molecule is the best characterized (June *et al.*, 1994). CD28 has two known ligands, B7-1 (CD80) and B7-2 (CD86) expressed primarily on APCs (Lenschow *et al.*, 1996). The interaction of IL-2 with its TCR constitutes the third signal in T cell activation, and leads to T cell differentiation (Sayegh and Turka, 1998).

1.1.1.3 Central role of CD4+ T cells

CD4+ T cells play a key role in orchestrating the various effector arms of the alloimmune response (reviewed in Hernandez-Fuentes *et al.*, 1999). Once fully activated, CD4+ T cells differentiate into either Th1 or Th2 effector cells (Mosmann and Sad, 1996). Th1 cells typically support delayed-type hypersensitivity (DTH) responses, the development of cytotoxic CD8+ cells and the production of some immunoglobulin (Ig)-G antibody (Ab) subclasses. Th1 cells are also involved in secretion of the cytokines; interferon (IFN)-γ, tumour necrosis factor (TNF)-β, and interleukin (IL)-2. Th2 cells, in contrast, support the production of other classes of Ab

and secrete the cytokines IL-4, IL-5 and IL-10. Cytokines play a central role in driving Th1 or Th2 expansion upon activation (Abbas *et al.*, 1996).

1.1.1.4 Effector mechanisms of rejection

A multitude of cell types recruited by CD4+ T cells are involved in allograft rejection. They include cytotoxic T lymphocytes (CTL), macrophages, natural killer (NK) cells and alloreactive Abs (produced by B lymphocytes) (reviewed in Arakelov and Lakkis, 2000).

1.1.1.4.1 Cytotoxic T lymphocytes

CTLs play an important role in allograft rejection by killing target cells which bear foreign major histocompatibility complex (MHC) (Liu *et al.*, 1996). In general, CTLs express CD8 molecules and recognize peptides presented by HLA class I molecules. CTLs kill donor target cells by at least two pathways. One pathway is mediated by the release of granules containing membrane poreforming proteins called perforins and a family of proteases called granzymes. Perforin forms large pores in the cell membrane and cause lysis, while granzymes enter the cytosol through perforin channels and induce cell death by apoptosis. Alternatively, CTLs can bind Fas on the target cell using their Fas ligand (FasL). Fas is a member of the TNF family of 'death receptors' which signal cell apoptosis (Nagata and Golstein, 1995).

1.1.1.4.2 Macrophages

Both CD4 T cells and activated macrophages can contribute to graft rejection by a DTH response (reviewed in Arakelov and Lakkis 2000). Activated CD4+ T cells stimulate macrophages via the CD40L-CD40 pathway and by secreting IFN-γ and lymphotoxin. Macrophages then release a host of mediators which include cytokines (TNF, IL-1, IL-6, IL-10, IL-12, and IL-15), chemokines, reactive oxygen species, nitric oxide, proteolytic enzymes, and extracellular matrix proteins that lead to fibrosis.

1.1.1.4.3 Natural killer cells

NK cells are a lymphocyte subset which do not express T- or B-lymphocyte markers. They are activated by IL-2, IL-15, and IFN-γ. NK cells appear to kill target cells by a process similar to CTL-mediated lysis, by releasing perforins and granzymes. Unlike CTLs, NK cells lack Ag-specific receptors. They express a low affinity receptor for the

constant (Fc) portion of IgG thereby binding to and killing Ab-coated target cells (Ab-dependent cell-mediated cytotoxcity or ADCC (Arakelov and Lakkis, 2000). The function of NK cells is regulated by a series of inhibitory receptors (Moretta *et al.*, 1996). These include members of the killer inhibitory receptor (KIR) family and the leukocyte Immunoglobulin-like receptors (LIR-1/ILT-2 and LIR-2/ILT-4) which bind HLA class I molecules. The inhibitory receptors, specific for HLA class I molecules, allow NK cells to discriminate between normal cells and cells that have lost the expression of HLA class I (e.g. tumor cells). As predicted by the "missing self hypothesis" (Ljunggren and Karre, 1990), NK cells recognize HLA class I molecules via surface receptors delivering signals that inhibit the NK cell function. Thus, NK cells lyse those target cells that have lost (or express insufficient amounts) of HLA class I molecules, a frequent event following tumour transformation and viral infection (reviewed in Moretta *et al.*, 2002). Most human NK cells also express a surface C-type lectin receptor CD94/NKG2A/B shown to interact with the nonclassical HLA class I molecule, HLA-E, to deliver an inhibitory signal to NK cells (Borrego *et al.*, 1998; Lee *et al.*, 1998) (see section 1.3.4).

1.1.1.4.4 Alloantibodies

Allogeneic HLA molecules are strong inducers of humoral responses. *In vivo*, serum Abs against HLA are induced by multiple blood transfusions, pregnancies, and also by previous transplants. These preformed alloantibodies mediate allograft damage in what is known as hyperacute rejection (discussed in section 1.1.2.1). Alloantibodies cause tissue damage by activating the complement cascade or by mediating ADCC (Rocha *et al.*, 2003).

Although the association between the presence of preformed circulating alloantibodies and hyperacute rejection has been well established (Terasaki, 2003), the clinical importance of donor specific antibodies (DSA) is surfacing. This has been made possible with development of objective diagnostic criteria, (Racusen, 2003) (see section 1.1.3.2) and the demonstration of the complement protein C4d in allograft biopsy as an important diagnostic tool (Feucht, 2003) (see section 1.1.3.2).

1.1.2 Clinical manifestations of graft rejection

Graft rejection has various time courses depending on the type of tissue or organ grafted and immune response involved. There are three patterns of graft rejection; hyperacute, acute and chronic rejection.

1.1.2.1 Hyperacute rejection

Hyperacute rejection usually appears within the first 48 hrs. This form of rejection is initiated by preformed donor-specific alloantibodies which bind to Ags on the graft's vascular endothelium. This subsequently leads to activation of complement, coagulation factors and other mediators of inflammation resulting in endothelial damage and vascular thrombosis and immediate graft loss (Arakelov and Lakkis, 2000). Preformed Abs which cause hyperacute rejection include two main groups, low affinity IgM Abs which are specific for the ABO blood group Ags, and high affinity IgG Abs targeted against class I HLA Ags (Williams *et al.*, 1968). Hyperacute rejection has been largely eliminated by ABO matching and by routine pre-transplant cross-matching whereby donor lymphocytes are mixed with the recipient's serum to detect the presence of cytotoxic Abs (Hernandez-Fuentes *et al.*, 1999).

1.1.2.2 Acute rejection

Rejection of solid organ grafts can still take place in the absence of any preformed Abs. In the clinical situation, with immunosuppression, this form of rejection occurs most frequently between 5 days and 3 months after transplantation (Hernandez-Fuentes *et al.*, 1999). T cells play a central role in the acute rejection process, although much controversy remains over the relative importance of the CD4+ and CD8+ T cell subpopulations in mediating transplant rejection. In some studies, CD8+ cells alone seem to be able to initiate allograft rejection (Hall, 1991), while in others, evidence suggests that CD4+ T cells are an absolute requirement (Kreiger *et al.*, 1996). In human biopsy studies there is also evidence to suggest that other molecules, including adhesion molecules and chemokines may also play an important role in acute rejection by facilitating the inflammatory process and mediating the recruitment of lymphocytes into the allograft (Fairchild *et al.*, 1997; Solez *et al.*, 1997).

Despite the well-documented role of cellular mechanisms in acute rejection, there is accumulating evidence to suggest that Ab-mediated immune mechanisms are significant in acute allograft rejection (reviewed by Mauiyyedi and Colvin, 2002). Trpkov *et al.* (1996) reported several specific morphological features in rejecting allografts that correlated with the presence of anti-class I Ab in the recipient. Ab-mediated graft rejection has however been difficult to diagnose due to the rapid removal of Ig from the surface of endothelial cells. Recently, the identification of C4d as a specific marker of Ab-mediated acute rejection has emerged (Feucht *et al.*, 1993; 2003; Regele *et al.*, 2001; Bohmig *et al.*, 2002). C4d is the stable remnant of classical complement activation, which binds covalently to the capillary wall, thereby providing *in situ* pathologic evidence of Ab-mediated injury. Pioneering studies by Feucht *et al.* (1993) reported capillary deposition of C4d, in 51 of 93 biopsies from renal allografts with early graft dysfunction. Among patients with C4d, 1-year survival was 57% compared with 90% survival in those without C4d. In 2001, the same group provided evidence of Abs producing early graft failures (Lederer *et al.*, 2001). They showed that the presence of C4d in 117 renal grafts led to significantly lower graft survival than in 101 grafts without C4d ($p=0.0001$). Later studies have confirmed a clear correlation of PTC C4d staining with concurrent circulating anti-donor-specific Ab and with certain pathologic features (Collins *et al.*, 1999; Mauiyyedi *et al.*, 2002). Capillary C4d is now incorporated in the 'Banff classification' for renal allografts (see section 1.1.3.2).

1.1.2.3 Chronic rejection

A significant complication in SOT is the progressive decline of allograft function that develops months or years after successful transplantation, commonly known as chronic rejection. The pathogenesis of chronic rejection remains elusive. For many years, chronic rejection has been considered to be immune mediated, driven by alloantigen-dependent mechanisms. However, alloantigen-independent risk factors have been identified as contributing to the development of chronic rejection. This will be discussed in section 1.1.3.2 as most of the research into chronic rejection has been carried out in renal transplantation.

1.1.3 Organ transplantation

1.1.3.1 Liver transplantation

In comparison with other organs such as the kidney, the liver is relatively resistant to hyperacute Ab-mediated rejection, as shown by the ability to carry out successful transplantation in the face of positive anti-donor cross-matching, including ABO-incompatibility (Gordon *et al.*, 1986). The basis for resistance to humoral rejection has been reviewed by Knechtle (1998). The most common cause of graft dysfunction is acute cellular rejection with an incidence of 50-80% (Wiesner *et al.*, 1998). It most commonly occurs between 4 and 14 days after transplantation (Neuberger, 1995). Histological examination of a liver biopsy specimen is considered the 'gold standard' for diagnosing acute rejection. An international consensus grading system called the Banff Schema for liver allograft rejection has been established for grading acute rejection (International Panel Comprised of Demetris, Batts, Dhillon *et al.*, 1997) (see table 1.1). Chronic liver allograft rejection, also termed vanishing bile duct syndrome (VBDS), develops slowly over a period of months or years and is a major cause of graft failure and retransplantation, although incidence is <5% (Knechtle, 1998). Diagnostic criteria for chronic rejection are (1) the presence of bile duct atrophy/pyknosis, affecting the majority of bile ducts, with or without bile duct loss; (2) convincing foam cell obliterative vasculopathy; or (3) bile duct loss affecting >50% of the portal tracts (Demetris *et al.*, 2000).

Grading of Acute Liver Allograft Rejection

Global assessment of rejection grade made on a review of the biopsy and after the diagnosis of rejection has been established.

Global Assessment*	Criteria
Indeterminate	Portal inflammatory infiltrate that fails to meet the criteria for the diagnosis of acute rejection (see reference below)
Mild	Rejection infiltrate in a minority of the triads, that is generally mild, and confined within the portal spaces
Moderate	Rejection infiltrate, expanding most or all of the triads
Severe	As above for moderate, with spillover into periportal areas and moderate to severe perivenular inflammation that extends into the hepatic parenchyma and is associated with perivenular hepatocyte necrosis

* Verbal description of mild, moderate or severe acute rejection could also be labeled as Grade I, II and III, respectively.

Table 1.1 Banff Schema for Grading Liver allograft Rejection: An International Consensus Document (International Panel Comprised of Demetris, Batts, Dhillon *et al.*, 1997).

1.1.3.2 Kidney transplantation

In renal transplantation, acute allograft dysfunction secondary to acute rejection occurs in 30-40% of patients (Chandraker, 1999). The 'gold standard' for diagnosis of acute rejection is histologic evaluation of a percutaneous needle biopsy of the transplant kidney. This has been aided by the development of an internationally standardized histological grading system known as the Banff 97 working classification (Racusen *et al.*, 1999). The Banff 97 working classification combined data from the previous Banff classification (Solez *et al.*, 1993) and the Collaborative Clinical Trials in Transplantation (Colvin *et al.*, 1997). In recent years, the Banff 97 classification of renal allograft rejection has been updated to include criteria for acute Ab-mediated rejection in renal allografts (see table 1.2) (Racusen *et al.*, 2003). Although in the majority of patients immunosuppressive agents may prevent or reverse most acute rejection episodes, the graft may still succumb to chronic rejection. More recently, the term chronic allograft nephropathy (CAN) was proposed because it was suggested that both immune and nonimmune factors may participate in the development of this progressive renal dysfunction (Tiley and Paul, 1996). The strongest evidence that chronic rejection is immune mediated stems from its association with acute rejection, and the degree of histocompatibility mismatching. In renal transplantation, acute rejection episodes that are either functionally or histologically more severe, and rejection episodes that are recurrent tend to be stronger predictors of chronic rejection (Tesi *et al.*, 1993). Chronic rejection is also less common in grafts that are better HLA matched, irrespective of whether they are from live-related or cadaveric donors (Cecka, 1998). In addition, HLA Abs has also been associated with chronic rejection and graft loss (McKenna *et al.*, 2000; reviewed in Terakasi, 2003). Recently, alloantigen-independent risk factors for chronic rejection have also been identified. These include donor source (living-related versus cadaveric), delayed graft function, size mismatching, donor age, donor and recipient gender, hyperlipidemia and hypertension (Ponticelli, 2000).

1.	Normal
2.	Antibody-mediated rejection Rejection due, at least in part, to documented anti-donor antibody ('suspicious for' if antibody not demonstrated); may coincide with categories 3, 4 and 5 Type (Grade) I. ATN-like – C4d+, minimal inflammation II. Capillary-margination and/or thromboses, C4d+ III. Arterial – v3, C4d+
3.	Borderline changes: 'Suspicious' for acute cellular rejection This category is used when no intimal arteritis is present, but these are foci of mild tubulitis (1-4 mononuclear cells/tubular cross-section) and at least i1 may coincide with categories 2 and 5
4.	Acute/active cellular rejection T-cell-mediated rejection; may coincide with categories 2 and 5 Type (Grade) Histopathological findings
	IA. Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of moderate tubulitis(>4 mononuclear cells/tubular cross-section or group of 10 tubular cells) IB. Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of severe tubulitis (>10 mononuclear cells/tubular cross-section or group of 10 tubular cells) IIA. Cases with mild to moderate intimal arteritis (v1) IIB. Cases with severe intimal arteritis comprising >25% of the luminal area (v2) III. Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)
5.	Chronic/sclerosing allograft nephropathy Fibrosing changes in the allograft, with or without features of true alloimmune injury to the graft; may coincide with categories 2, 3 and 4. Grade Histopathological findings
	Grade I (mild) – Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changed suggesting chronic rejection Grade II (moderate) – Moderate interstitial fibrosis and tubular atrophy (a) or (b) Grade III (severe) – Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b)
6.	Other Changes not considered to be due to rejection; may coincide with categories 2, 3, 4 and 5

Table 1.2 Banff 97 diagnostic categories for renal allograft biopsies – update (Racusen *et al.*, 2003).

1.1.4 Immunosuppressive therapy

The main aim of transplantation is the ultimate survival of the graft in the transplant recipient, and where possible to ensure early engraftment of the organ with no rejection. Graft rejection can be minimized by cross-matching donor and graft for ABO and HLA tissue types, however, this is not always possible due to the shortage of available donor organs for transplantation. To prevent rejection of the transplanted organ or bone marrow, immunosuppression is therefore required. This can be achieved through the use of immunosuppressive drugs.

1.1.4.1 Pharmacological drugs

The current immunosuppressive drugs include cyclosporin A, tacrolimus (FK506), prednisolone, azathioprine and a preparation of anti-T-cell Abs. They act to inhibit the various steps of the T-cell activation pathway (table 1.3 shows the mechanism of action of these drugs and side effects). Immunosuppressive drugs are effective in reducing acute rejection. However, their potential adverse effects and contribution to increased risk of infectious complications in transplant recipients, means their use must be reduced to a manageable level. Currently, a combination of immunosuppressive drugs has proved to be an effective strategy to inhibit diverse pathways involved in allograft rejection, and allowing the reduction of both dosage and adverse effects of each individual drug. Many centers have administered a combination of cyclosporin, prednisolone and azathioprine, often referred to as 'triple therapy'.

Agent	Mode of Action	Side-Effects
ATG/ALG	Binds multiple antigens on lymphoid cells, resulting in modification of cell surface receptors or complement mediated lysis	Serum sickness Thrombocytopenia Leucopenia
OKT3 Ab	Binds T cell CD3	Cytokine release syndrome Increase risk of PTLD
IL-2 receptor antibody	Block cytokine gene transcription via inhibition of NFAT	Glucose intolerance Hypertension Hyperlipidemia Growth Delay Osteopenia
Cyclosporin	Inhibit action of calcineurin, thereby inhibiting production of IL-2	Hypertension Nephrotoxicity Hirsuitism, gum hypertrophy
Tacrolimus (FK506)	Inhibit action of calcineurin, thereby inhibiting production of IL-2	Glucose intolerance Hypertension Nephrotoxicity
Azathioprine	Inhibits purine (adenosine) synthesis	Bone marrow suppression Transaminitis
Mycophenolate mofetil	Inhibits purine (guanosine) synthesis	Bone marrow suppression Gastrointestinal upset
Rapamycin (Sirolimus)	Blocks IL-2 induced cell cycle progression	Hyperlipidemia Leukopenia, thrombocytopenia

Table 1.3 Current Immunosuppressants: Mode of Action and Associated Side Effects (Marion, 2003). Abbreviations: ATG anti-thymocyte globulin; ALG anti-lymphocyte globulin; NFAT nuclear factor of activated T-cells; PTLD post-transplantation lymphoproliferative disease.

1.1.5 Infections following solid organ transplantation (SOT)

The occurrence of infection in SOT is a major determinant of transplant outcome (reviewed by Patel and Paya, 1997; and Fishman and Rubin, 1998). Infection is closely related to the degree of immunosuppression, and thus to the frequency and intensity of rejection and its therapy. There are three time frames, influenced by surgical factors, the level of immunosuppression, and environmental exposures, during which infections of specific types most frequently occur post-transplantation. These include the first month; one to six months, and more than six months after transplantation (see figure 1.1).

Most infections that occur in the first month post-transplantation are related to surgical complications. These include bacterial and candidal wound infections. From the second to sixth month post-transplantation period, infections are usually a result of opportunistic pathogens (HCMV, Epstein Barr virus (EBV), other human herpesviruses, hepatitis B and C viruses (HBV and HCV) and human immunodeficiency virus (HIV). From six months post-transplantation onwards most transplant recipients do relatively well, suffering from the same infections seen in the general community, namely influenza virus infection, urinary tract infection and pneumoccocal pneumonia. The only opportunistic viral infection commonly seen during this period is reactivated varicella zoster virus (VZV). However, two conditions can predispose patients to infection during this late post-transplantation period. Firstly, patients who have frequent episodes of acute rejection requiring augmented immunosuppression, or those with chronic rejection who are maintained at a higher baseline level of immunosuppression, are at increased risk of opportunistic infections typically seen in the second to six month post-transplantation period. Secondly, patients who have chronic or progressive infection with HBV, HCV, HCMV, and uncommonly HIV, may suffer from morbidity associated with these agents which may lead to an increase in other infections. Although evidence is far from being conclusive, several studies have suggested that herpesvirus infections could perhaps trigger allograft rejection in different transplant settings. The evidence linking these viruses, particularly HCMV, HHV-6 and -7 to allograft rejection will be examined in later sections.

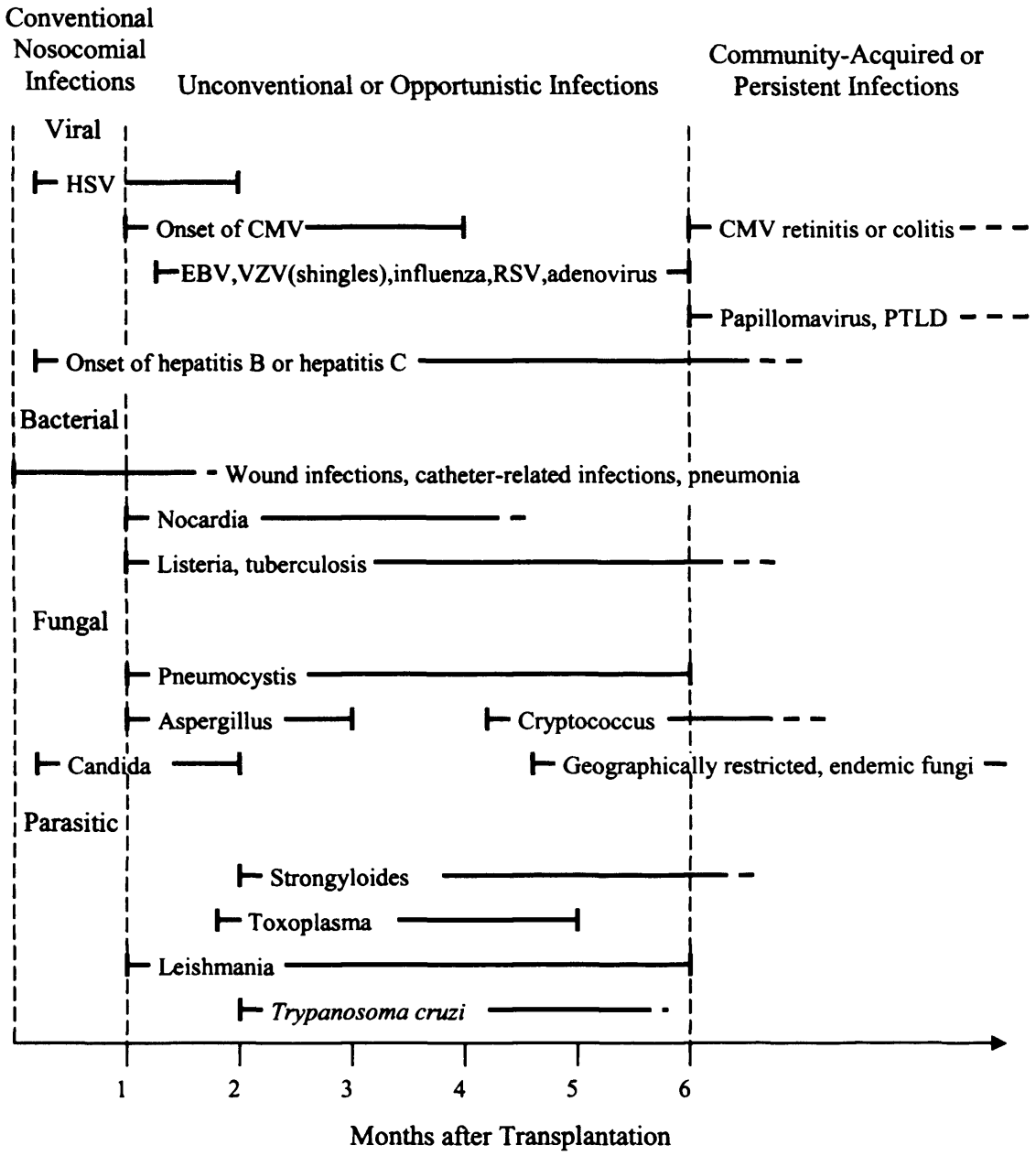


Figure 1.1 Usual sequence of infections after Organ Transplantation (Fishman and Rubin, 1998). Exceptions to the usual sequence of infections after transplantation suggest the presence of unusual epidemiologic exposure or excessive immunosuppression. Abbreviations: HSV denotes herpes simplex virus; CMV cytomegalovirus; EBV Epstein-Barr virus; VZV varicella zoster virus; RSV respiratory syncytial virus; and PTLD post-transplantation lymphoproliferative disease. Zero indicates the time of transplantation. Solid lines indicate the most common period for the onset of infection; dotted lines and arrows indicate periods of continued risk at reduced levels.

1.2 The herpesviruses

The family *Herpesviridae* are made up of a group of more than 100 viruses, which infect and cause a variety of diseases in most animal species. Eight human herpesviruses have been discovered to date.

1.2.1 Herpesvirus structure

Under the electron microscope, all herpesviruses appear identical. Typically the herpesviruses consists of a core, which contains the linear double-stranded DNA and a 100-110 nm icosadeltahedral capsid containing 162 capsomeres, 12 pentameric at the vertices and 150 hexameric. The capsid is in turn surrounded by an amorphous layer of proteins called the tegument, which may vary in thickness, depending on the location of the virion within the cell. Surrounding the tegument is the lipid envelope. It has a trilaminar appearance, suggestive of it being derived from patches of altered cellular membranes. The outer surface of the envelope contains numerous protrusions of glycoprotein, which appears to be acquired from the host by budding through the nuclear membrane (figure 1.2).

In addition to the morphological properties described so far, the herpesviruses share many significant biologic properties. All herpesviruses encode a large range of enzymes involved in nucleic acid metabolism, DNA synthesis and processing of proteins (i.e. kinase activity). The synthesis of viral DNAs and the assembly of capsids occur in the nucleus, and the release of progeny virus invariably results in the destruction of the host cell. The herpesviruses also have the ability to remain latent within the natural host. In the cells harbouring latent virus only a subset of viral genes, if any are expressed.

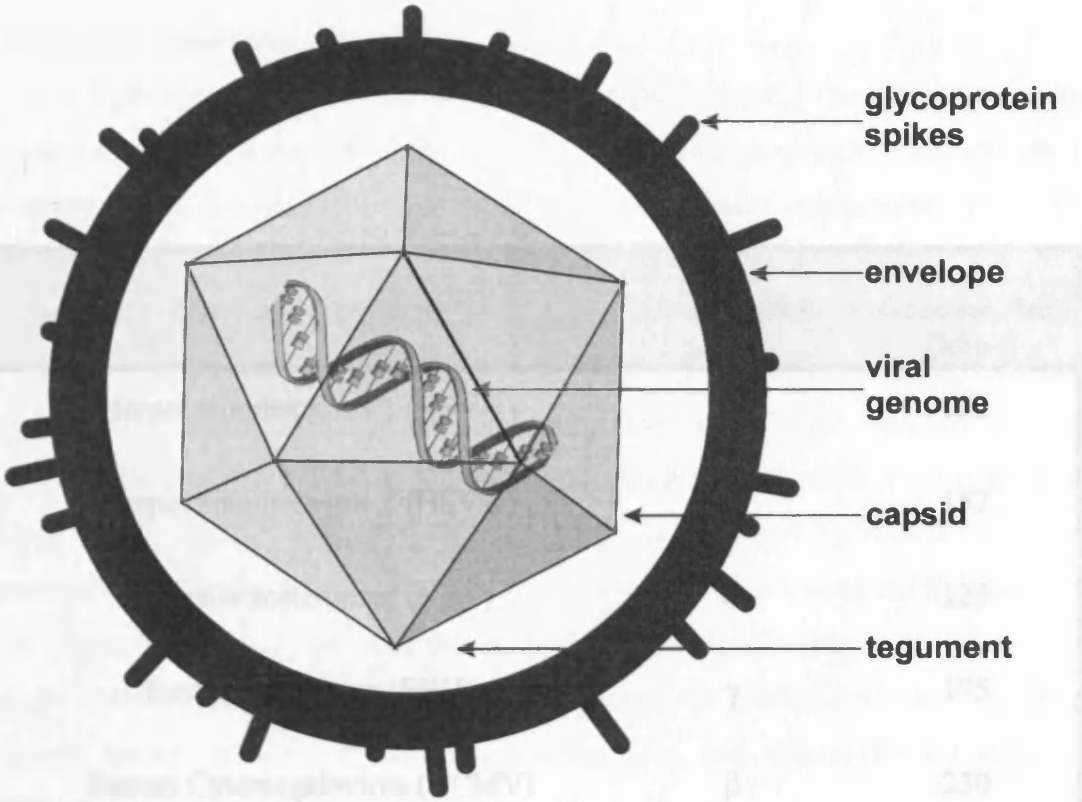


Figure 1.2 Schematic showing the major structural components of a herpesvirus particle.

1.2.2 Herpesvirus classification

In the past, the classification of the Herpesviruses was predominantly based upon their biological properties such as host range and cell tropism, cell pathology in laboratory culture, and cellular sites of latent infection. The international Committee on the Taxonomy of Viruses (ICTV) has classified the herpesviruses into three subfamilies, the *α-herpesvirinae, β-herpesvirinae*, and *γ-herpesvirinae,* on the basis of these biological properties. Table 1.4 shows the classification of the human herpesviruses. Although the classification of Herpesviruses on biological characteristics is useful, it is subjective and does produce anomalies e.g. EBV, a γ-herpesvirus, is lytic in some cell types like the α-herpesviruses, but lymphotropic like the β-herpesviruses. In addition, recent advances in molecular biology have led to the discovery of new herpesviruses, HHV-8, before it could be propagated in cell culture. Therefore, classification of the viruses is now based on genome analysis of the human herpesviruses, with parameters such as similarities of genomic organization, homology between nucleotide and amino acid sequences taken into account.

Human Herpesvirus	Classification	Genome size (kbp)
Herpes simplex virus 1 (HSV-1)	α	152
Herpes simplex virus 2 (HSV-2)	α	152
Varicella zoster virus (VZV)	α	125
Epstein-Barr virus (EBV)	γ	175
Human Cytomegalovirus (HCMV)	β	230
Human herpesvirus 6 (HHV-6)	β	160-162
Human herpesvirus 7 (HHV-7)	β	145
Human Herpesvirus 8 (HHV-8) (Kaposi's sarcoma-associated herpes virus)	γ	165-170

Table 1.4 Classification of the Human Herpesviruses.

1.2.3 Herpesvirus life cycle

The kinetics of herpesvirus replication has been most intensely investigated for HSV, and general mechanisms are similar throughout the other subfamilies. However, there are significant differences among the various subfamilies and amongst members within the same subfamily.

1.2.3.1 Attachment and penetration

Prior to replication, the virus must gain entry into the host cell. The virus attaches itself to the cell surface via the interaction between its surface glycoprotein's and specific cell receptors. This is mediated by a number of receptor/ligand interactions. For HSV-1 and HCMV, glycoprotein's such as gB, gD (not HCMV), gH and gL are required. In HSV-1 replication the initial interaction involves the viral envelope gC, and to a lesser extent gB, with the glycosaminoglycan moieties of cell surface heparan sulphate (Shieh *et al.*, 1992). The second step is followed by association of gD with one of several cellular molecules that belong to three different classes of cell-surface receptor families (Spear *et al.*, 2000). Entry into the cytoplasm is mediated by fusion of the viral envelope with the plasma membrane of the host cell. This requires the intervention of four glycoproteins, gD, gB, and the heterodimer gH-gL (Turner *et al.*, 1998). The capsid is then transported along the cytoskeleton to the nucleus, where viral DNA is released, enters the nucleus through a nuclear pore, and circularizes (Garber *et al.*, 1993).

1.2.3.2 Genome replication

Productive infection of the herpesviruses follows a highly co-ordinated cascade of gene expression (see figure 1.3). Three classes of messenger RNA (mRNA) are transcribed in a highly co-ordinated fashion by the cellular RNA polymerase II; the immediate early (IE), early (E) and late (L) genes (or α, β and γ genes). The HSV IE genes expressed consist of six proteins designated as ICP0, ICP4, ICP22, ICP27, ICP47, and Us1.5, most of which are transactivators. Although transcription of the α genes requires no prior viral protein synthesis a tegument protein, VP16 in HSV-1 (also called α-TIF (α-gene transcription-inducing factor) augments the transcription of the α genes. The tegument protein associates with other cellular proteins to form a complex which upregulates cellular RNA polymerase activity by binding to the IE promoter enhancer region of viral DNA. After transcription, the α-mRNAs are transported to the cytoplasm where they are translated into regulatory proteins. These regulatory proteins control the expression of all later genes, in particular the β-genes, the products of which include enzymes involved in viral DNA replication and nucleic acid metabolism, and depending on the virus include ribonucleotide reductase, thymidine kinase, helicase primase and viral DNA polymerase. Viral DNA replication follows, by the rolling circle mechanism, where complete head-to-tail concatamers of viral genomes are formed.

Following DNA replication, β proteins induce the switch from transcription of β genes to γ genes and the resulting 'late' mRNAs are translated into γ-proteins, most of which have structural roles in the assembly of the virion.

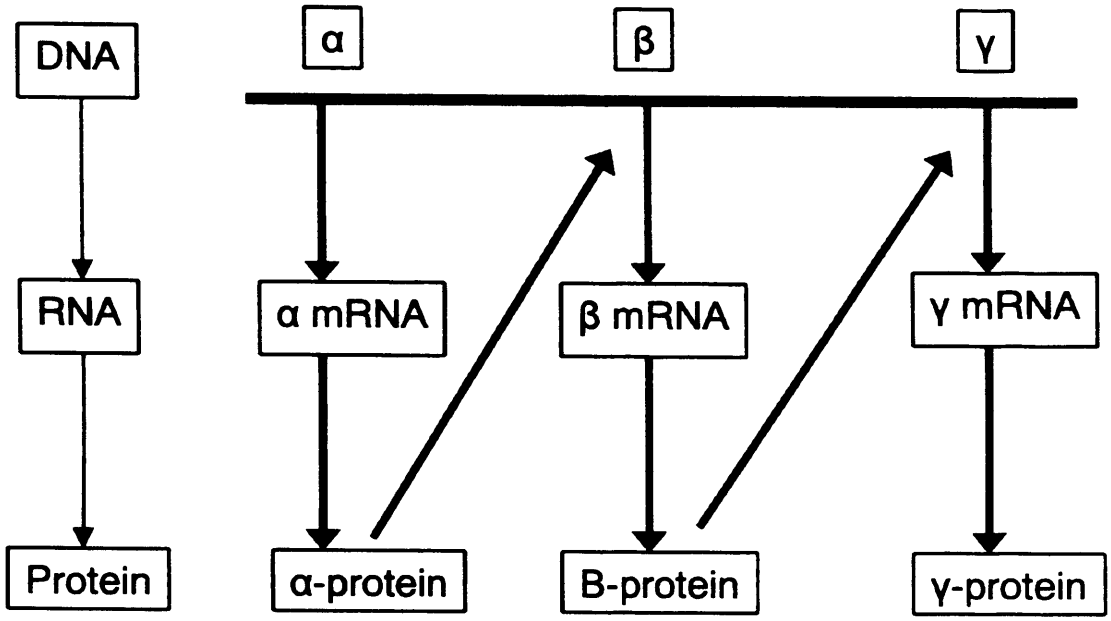


Figure 1.3 Cascade genome expression of herpesviruses (Griffiths, 2000). Genes labeled α (immediate-early), β (early) or γ (late) are transcribed into messenger RNA and then translated into proteins.

1.2.3.3 Assembly and budding

Capsid proteins associate to form empty capsids in the nucleus, and genome unit-length viral DNA cleaved from the concatamers is packaged to produce nucleocapsids, which then associate with patches of nuclear membrane to which tegument and glycosylated envelope proteins have bound. Envelopment of the capsids is achieved by budding through the inner nuclear membrane. The route for egress of the virion particle from the space between the inner and outer nuclear membranes to the exterior of the infected cell is controversial. It may occur by either of two general pathways (reviewed in Roizman and Knipe, 2001). In pathway A, often called the re-envelopment pathway, the envelope fuses with the outer nuclear membrane, de-enveloping the capsid and releasing

it into the cytoplasm. The capsid then buds into the Golgi apparatus (GA), forming an enveloped virion, which is transported to the surface by vesicular transport. In pathway B, called the luminal pathway, the virion particle buds through the outer nuclear membrane and is transported by vesicular movement through the GA to the exterior of the cell.

1.2.4 Viral persistence

Following primary infection, all herpesviruses can persist in the host. This may take the form of latency or chronic active replication. During latent infection, infectious progeny are not produced, and viral gene expression is limited to a small subset of viral genes required for maintenance of latency, the number of which varies among viruses. Reactivation from latency with concomitant virus production is an efficient means by which the virus can spread within the host population. Each human herpesvirus has evolved its own unique ecological niche within the host that allows the maintenance of latency over the life of the individual (see table 1.5). Primary HSV-1 infection is often an inapparent asymptomatic infection of the mouth and lips or can cause herpes labialis, after which the virus becomes latent in dorsal root ganglia. Reactivation of latent HSV-1 can be asymptomatic or result in localized epithelial eruptions (cold sores) that usually resolve in a few days. During the latent phase of HSV-1 infection, viral gene expression is highly restricted. Only the latency associated transcripts (LAT) are produced in abundance during latency. The unprocessed 8.3 kb LAT message maps to the opposite strand encoding the IE HSV-1 gene, ICPO (Zwaagstra *et al.*, 1990). While mutations that eliminate a large portion of the HSV-1 LAT still establish and reactivate from latency (Javier *et al.*, 1988), reduced levels of LAT transcripts have been associated with a reduced efficiency of reactivation (Perng *et al.*, 1994). HCMV and HHV-6 establish latent infection in monocyte/macrophage lineage, and during latent infection both viruses also express LAT that show similar features: (i) both transcripts contain open reading frames (ORFs) encoding IE proteins IE-1 and IE-2 (Kondo *et al.*, 2002a; 1996) and both transcripts are expressed in a small proportion of latently infected cells (Kondo *et al.*, 1991; Slobedman and Mocarski, 1999). However, it is unclear if these LAT are required for latency or reactivation (Lunetta and Wiedeman, 2000). In a recent study, HHV-6 LATs were found to be abundantly expressed at an immediate stage between latency and reactivation. HHV-6 LATs functioned as sources

of IE-1 protein at this stage, which up-regulated the viral reactivation (Kondo *et al.*, 2003).

Sub-family name	Cellular site of latency
Herpes simplex virus type 1	neurons
Herpes simplex virus type 2	neurons
Varicella Zoster Virus	neurons
Epstein Barr Virus	B lymphocytes
Human Cytomegalovirus	monocytes
Human Herpesvirus 6	T cells/monocytes
Human Herpesvirus 7	T cells/monocytes
Human Herpesvirus 8 (Kaposi's sarcoma associated herpes virus)	B cells, endothelial cells?

Table 1.5 Human herpesviruses and the sites in which they establish latency.

1.3 Human Cytomegalovirus (HCMV)

HCMV is the prototype member of the β-herpesvirus subfamily. HCMV infects approximately 60% of adults in developed countries and more than 90% of adults in the developing world. In the immunocompetent individual, infection is usually asymptomatic. Occasionally, however, primary infections can produce a self-limited mononucleosis syndrome. After primary infection the virus is not eliminated from the body, but persists lifelong including a latent state. However, when the host's immune system is compromised as a result of infection (e.g. HIV), immaturity (neonates) or iatrogenic immunosuppression in SOT or bone marrow transplantation (BMT), the virus replicates exerting its full pathogenic potential.

In SOT recipients the incidence of active HCMV infection ranges from 23% to 85% and symptomatic disease develops in approximately half of these patients (Kanj *et al.*, 1996). HCMV infection in SOT recipients exhibits a wide range of disease manifestations from asymptomatic infection to severe, lethal HCMV disease. In addition to the direct pathogenicity on end-organs, HCMV has been associated with increased incidence of opportunistic bacterial and fungal infections, accelerated atherosclerosis, and allograft rejection, collectively they are known as the 'indirect effects' of HCMV (Rubin, 1989) (described in sections 1.3.9).

1.3.1 Discovery

HCMV was first discovered in the 1950s when investigating the pathological features of cytomegalic inclusion disease (CID), a syndrome affecting neonates and infants. In 1956, three groups (Smith, Rowe and Weller) subsequently isolated HCMV independently. The AD169 laboratory strain was the first to be completely sequenced. Analysis of its 230 kbp genome has revealed that it encodes 225 ORFs of approximately 100 or more amino acids (Chee *et al.*, 1990). However, laboratory-adapted strains of HCMV have extensive gene deletions compared to wild-type strains and so do not accurately represent the authentic virus which infects humans (Cha *et al.*, 1996; Dolan *et al.*, 2004). Recently, a low passage strain (Merlin) has been sequenced and its genetic content has been found to be close to the authentic HCMV genome (Dolan *et al.*, 2004).

1.3.2 Propagation and Cell tropism

HCMV exhibits a highly restricted host range in cell culture. *In vitro*, primary differentiated human fibroblasts from skin or lung are the only cells fully permissive for replication of laboratory strains, whereas clinical isolates replicate preferentially in endothelial cell cultures. In fibroblasts, the replication cycle is slow, with a typical cytopathic effect (CPE) characterized by cell enlargement and rounding with both intra- and perinuclear inclusions. In contrast, during natural infection, HCMV has been found to be capable of replicating in a wide variety of cell types including fibroblastic, epithelial, macrophage, smooth muscle, and endothelial cells (Sinzger *et al.*, 1995; 1996). Latent viral DNA, thus, can be detected in macrophage-granulocyte progenitors in the bone marrow and in peripheral monocytes (Kondo *et al.*, 1994; Soderberg-Naucler *et al.*, 1997). The replication rate of HCMV *in vivo* has been shown by the frequent measurement of HCMV load in the blood following antiviral intervention (Emery *et al.*, 1999a). This study demonstrated that, during an active HCMV infection, the doubling time of HCMV is approximately one day; thus HCMV replication occurs dynamically in the host, contrasting to the results obtained from *in vitro* propagation (Emery *et al.*, 1999a).

1.3.3 HCMV and immunomodulation

A characteristic feature of HCMV infection in the normal host is persistence of the virus in a latent state at specific anatomical sites for months or years. Recurrent infections may occur when the host becomes immunocompromised. This ability to avoid elimination by the immune system is the result of (1) induction of a latent state of infection where the virus does not adversely affect the host but restricts the number of viral genes expressed so as to minimize exposure to the immune system, (2) exploitation of immunologically privileged sites for replication (i.e. epithelial cells of the salivary glands). These cells do not express sufficient HLA class I molecules to trigger viral clearance by CD8+ cell, (3) expression of genes that interfere with the immune response (reviewed in Mocarski, 2002; Landolfo *et al.*, 2003).

This section will focus on the means by which HCMV can evade the host's immune system. Escape from cell-mediated immunity is mediated by several mechanisms. HCMV phosphoprotein (pp)65 (UL83) a tegument protein, inhibits Ag processing by phosphorylating the 72-kDa IE protein (one of the first viral proteins produced in the infected cell) leading to a lack of processing within the proteosome (Gilbert *et al.*, 1996). HCMV encodes four proteins- glycoprotein (gp) US2, gpUS3, gpUS6 and gpUS11 which independently decreases cell-surface expression of HLA class I proteins (Ahn *et al.*, 1997; Jones *et al.*, 1996; Wiertz *et al.*, 1996).

Downregulation of HLA class I expression on infected cells is thought to render the cell susceptible to NK cell killing, because cells require presentation of the signal peptides derived from HLA class I A, B, or C to be protected against NK cell lysis (Borrego *et al.*, 1998). However, the HCMV genome contains an HLA class I homologue (UL18) (Fahnestock *et al.*, 1995), which exhibits high affinity for LIR-1/ILT-2 (Chapman *et al.*, 1999). The function of gpUL18 during virus infection is not yet resolved. One study reported protection against NK cell lysis (Reyburn *et al.*, 1997), whereas in another study, expression of gp UL18 was shown to enhance NK cell killing (Leong *et al.*, 1998). As mentioned in section 1.1.1.4.3, NK cells also express a surface C-type lectin receptor CD94/NKG2A/B which interacts with the nonclassical HLA class I molecule, HLA-E to deliver inhibitory signal to NK cells. HLA-E exhibits limited polymorphism and binds a restricted set of peptides derived from the leader sequence of classical HLA class I molecules and HLA-G (Lee *et al.*, 1998; Llano *et al.*, 1998). Class I leader peptides are released in the cytoplasm and then transported by a transporter associated with Ag processing (TAP) into the lumen of the endoplasmic reticulum (ER), where it binds to HLA-E in a fashion similar to that of the classical HLA molecules (Lee *et al.*, 1998). It has been shown that the HLA-E binding peptide is contained within the leader sequence of HCMV gpUL40. Independently of TAP, gpUL40 can up-regulate expression of the HLA-E, which protects targets from NK cell lysis (Tomasec *et al.*, 2000).

HCMV contains four ORF with homology to G-protein-coupled receptors (GPCR) (UL33, UL78, US27 and US28) which may act as receptors for chemokines (Chee *et al.*, 1990). The US28-encoded GPCR pUS28 is capable of binding CC chemokines MIP-1α (macrophage inflammatory protein 1α), MCP-1 (monocyte chemotactic protein-1), and RANTES (regulated upon activation, normal T expressed and secreted) (Bodaghi *et al.*, 1998), as well as soluble forms of the CX3C chemokine fractalkine (Kledal *et al.*, 1998). In accordance with its chemokine binding activity, pUS28 was suggested to be responsible for CC chemokine sequestration in HCMV-infected cells, thereby eliminating these chemoattractants from the environment of these cells *in vivo* (Bodaghi *et al.*, 1998). Furthermore, upon interaction with the CC chemokines, pUS28 induces intracellular calcium mobilization (Vieira *et al.*, 1998). Thus, this receptor functions as a 'chemokine sink', possibly enabling HCMV-infected cells to evade immune surveillance (Randolph-Habecker *et al.*, 2002). In addition, the expression of US28 in smooth muscle cells (SMCs) in the presence of MCP-1 and RANTES promoted SMC migration (Streblow *et al.*, 1999). This provides evidence linking HCMV to the acceleration of vascular diseases, such as restenosis, SOT vascular sclerosis and atherosclerosis (Streblow *et al.*, 1999).

Two genes (UL146 and UL147) within the HCMV genome exhibit weak amino acid homology to the CXC chemokines. UL146 encodes an IL-8 like chemokine, denoted vCXC-1 (Penfold *et al.*, 1999). The UL146 stimulates neutrophil migration, thus possibly aiding the dissemination of the virus. HCMV also encodes a viral IL-10 homologue (Kotenko *et al.*, 2000). Analogous to host IL-10, HCMV IL-10 reduces cell-surface expression of HLA class I and class II proteins on peripheral blood mononuclear cells (PBMCs) (Spencer *et al.*, 2002).

1.3.4 Epidemiology and Transmission

Primary infection with HCMV occurs mostly during childhood. In developed countries HCMV seropositivity varies from 30% to 70% (approaching >70% in older populations and in the lower socioeconomic groups) and is near 100% in developing countries (Griffiths, 2000). HCMV can be acquired by intrauterine infection, perinatal transmission from mother to child either during delivery following ingestion of infected maternal genital secretions or by ingestion of breast milk containing HCMV (Stagno *et al.*, 1980) (5-20%), or by close contact between individuals through exposure to

infected bodily fluids such as saliva, tears, urine and semen. In addition to natural routes of transmission, HCMV can be transmitted by iatrogenic means, such as blood transfusion (Adler *et al.*, 1983) or following organ transplantation in the donor organ (Pass *et al.*, 1983).

1.3.5 Congenital HCMV infection

Around 5% to 10% of congenitally infected babies are born with symptoms characteristic of what was initially termed CID. The clinical findings usually include one or more of the following; low birth weight, petechial haemorrhages, jaundice, hepatosplenomegaly, microcephaly, encephalitis, and occasionally chorioretinitis, and inguinal hernia. Congenital HCMV infection is important medically as it can cause permanent neurological damage and accounts for the long-term morbidity and poor prognosis of HCMV inclusion disease (Boppana *et al.*, 1992a).

1.3.6 HCMV infection in children and adults

Primary infection in adolescence and adults is usually subclinical or mild, but nonetheless, is thought to account for 8% of all cases of mononucleosis. (Nesmith and Pass, 1995). Clinical manifestations of mononucleosis due to HCMV are very similar to that caused by EBV although lymphadenopathy is uncommon.

1.3.7 HCMV infection in transplant recipients

In the immunocompromised host, HCMV exhibits a wide range of disease manifestations. Most cases of HCMV disease present with a non-specific syndrome which manifests as fever, malaise, neutropenia, thrombocytopaenia, and elevated liver enzymes in serum, such as transaminases. These symptoms may be mild and temporary or may progress to more severe end organ disease. Organ involvement by HCMV varies amongst different immunocompromised patient groups e.g. hepatitis is frequently diagnosed in liver transplant recipients, whereas pneumonitis is the major disease in bone marrow recipients (table 1.6).

HCMV infection can be transmitted to transplant recipients from one of three routes 1) primary infection occurs when a CMV-seronegative recipient becomes infected with latent virus from an organ or from virus carried in blood products from the seropositive donor; 2) secondary or reactivation infection occurs when endogenous latent virus is reactivated in a seropositive recipient; 3) reinfection occurs when a seropositive recipient receives latent virus from the seropositive donor and the virus that reactivates post-transplantation is of donor origin. Symptomatic disease occurs most frequently in patients experiencing primary infection.

Symptoms	Solid organ transplant	Bone marrow transplant	AIDS
Fever/hepatitis	++	+	+
Gastrointestinal	+	+	+
Retinitis	+	+	++
Pneumonitis	+	++	
Myelosuppression		++	
Encephalopathy			+
Polyradiculopathy			+
Addisonian state			+
Immunosuppression	+		
Rejection/GVHD	+	?	

Table 1.6 HCMV diseases in the immunocompromised (Griffiths *et al.*, 2000).

Abbreviation: GVHD graft verses host disease

+, Diseases that occur in the designated patient population

++, Diseases that were the most common

?, Suggested, but not proven association

1.3.8 Diagnosis of HCMV disease

HCMV infection and disease are important causes of morbidity and mortality among transplant recipients. As mentioned above HCMV causes a range of diseases in immunocompromised patient groups, and so for the purpose of developing consistent reporting of HCMV in clinical trials, definitions of HCMV infection and disease has been internationally agreed (Ljungman and Plotkins, 1995; Ljungman *et al.*, 2002). A case definition for HCMV disease requires the patient to have compatible clinical syndrome consisting of both symptoms and signs, together with the detection of HCMV in biopsies of the affected organ (with the exception of the retina, where the characteristic clinical appearance is sufficient) (see table 1.7).

Disease	Definition
Pneumonia	Signs and/or symptoms of pulmonary disease Detection of CMV in BAL fluid or lung tissue sample
Gastrointestinal disease	Clinical symptoms developed in the upper or lower gastrointestinal tract Macroscopic mucosal lesions on endoscopy Demonstration of CMV infection (by culture, histopathological testing, IHC or ISH) in a gastrointestinal tract biopsy specimen
Hepatitis	Elevated bilirubin and/or enzyme levels during liver function testing Absence of any other documented cause of hepatitis Detection of CMV infection in a liver biopsy specimen
CNS disease	Identification of CNS symptoms Detection of CMV in CSF samples, by culture or PCR, or in brain biopsy specimens
Retinitis	Typical ophthalmological lesions without virological proof

Table 1.7 Definitions of Cytomegalovirus end organ disease (updated from Ljungman *et al.*, 2002). Abbreviations: BAL, bronchoalveolar lavage; CNS, central nervous system; CSF, cerebrospinal fluid; ISH, *is situ* hybridisation; IHC, immunohistochemistry.

1.3.8.1 Risk factors for HCMV disease

Primary infection is one of the risk factors for the development of HCMV disease for recipients of SOT but not for BMT recipients. Studies have shown that primary infection acquired from a seropositive donor is associated with a high risk of HCMV disease, whereas reactivation of the recipient's latent virus has a lower risk of disease (Betts *et al.*, 1977; Grundy *et al.*, 1988, Ranjan *et al.*, 1991). Re-infection (donor virus infecting an immune recipient) is associated with an intermediate risk of disease (Grundy *et al.*, 1988). Viraemia has also been shown to be a risk factor for HCMV disease (Pillay *et al.*, 1993; Kidd *et al.*, 1993).

The importance between the quantity of HCMV and the development of HCMV disease was first reported in 1975 by Stagno and coworkers, who showed that high HCMV titres in the urine were significantly associated with disease in congenitally infected infants. More recently using molecular methods the importance of HCMV virus load in liver transplant (Cope *et al.*, 1997a), renal transplant (Cope *et al.*, 1997b), and BMT (Gor *et al.*, 1998) patients has been shown. Furthermore, multivariate analyses showed that once virus load had been controlled for, other recognized risk factors of donor/recipient (D/R) serostatus and the detection of HCMV in the blood by PCR (DNAemia) were no longer statistically associated with HCMV disease (Cope *et al.*, 1997a; 1997b; Gor *et al.*, 1998; Hassan-Walker *et al.*, 1999). These results demonstrate that high HCMV load is the major determinant of HCMV disease, and that previously recognized risk factors of DNAemia and donor/recipient serostatus are markers of HCMV disease simply because of their correlation with a high virus load. In addition, Cope *et al.* (1997a) showed in a multivariate analysis that receipt of methylprednisolone to control rejection episodes in liver transplant recipients is a risk factor for HCMV disease, independent of high viral load. Thus, the administration of methylprednisolone seemed to make a patient more susceptible to disease at a lower peak HCMV load. This effect appears to be particular to methylprednisolone because association between the increased risk of HCMV disease and the administration of anti-thymocyte globulin (ATG) can be explained wholly by raised HCMV load (Hassan-Walker *et al.*, 1999).

1.3.9 Indirect effects of HCMV

In addition to the direct end organ diseases discussed above, HCMV has been associated with indirect effects including the virus' contribution to the net state of immunosuppression thus increasing the risk of bacterial and fungal infections, and acute and chronic allograft rejection in different transplant settings (reviewed in Cainelli and Vento, 2002; Lautenschlager, 2003).

1.3.9.1 Secondary infections

One of the most important effects of HCMV infection after transplantation is predisposition to life-threatening superinfection with a variety of microbial agents including *Listeria monocytogenes, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus* species (Rubin *et al.*, 1977; Chatterjee *et al.*, 1978; George *et al.*, 1997). However, in a recent multicenter study, HCMV was not found among the risk factors predicting invasive candidiasis in liver transplant patients (Husain *et al.*, 2003)

Recently, associations between HCMV and other viral infections have been reported. In HCV-infected liver transplant recipients who reactivate HCMV, higher rates of allograft failure and mortality were found compared to those who do not reactivate HCMV (Razonable *et al.*, 2002a). The causal relationship between HCMV and opportunistic infections can be demonstrated in randomized trials with antiviral agents which suppress HCMV replication. In a recent study by Lowance *et al.* (1999), antiviral prophylaxis with valaciclovir (VACV) was found to significantly reduce the incidence of HCMV infection and disease, and nonherpesvirus infections such as *Candida* (10% VACV versus 22% placebo) and staphylococcal infections (12% versus 21%) in renal transplant recipients. Wagner *et al.* (1995) reported ganciclovir (GCV) prophylaxis reduced the risk for fungal infections compared with placebo (7% versus 27%) in heart transplant recipients.

1.3.9.2 HCMV and atherosclerosis

In 1970, Richard Simmons and coworkers first suggested a relationship between acute and chronic allograft injury and HCMV infection. Since then, there has been considerable controversy over this putative relationship. The most convincing evidence for an association between HCMV infection and allograft-related pathology stems from cardiac transplantation. Several groups reported that HCMV infection is related to enhanced cardiac allograft vasculopathy (chronic rejection) (Grattan *et al.*, 1989; McDonald *et al.*, 1989; Loebe *et al.*, 1990), and classical atherosclerosis (Melnick *et al.*, 1990; Hendrix *et al.*, 1989). These findings have been confirmed in studies of endomyocardial biopsies where an association between CMV antigenemia and a mild inflammatory cell response in the vessel wall, with alterations in small intramyocardial arterioles leading to a narrowing of the vascular lumen of the graft was demonstrated (Koskinen *et al.*, 1993a: 1993b). In an autopsy study of three heart allograft recipients with severe graft arteriosclerosis, HCMV nucleic acids were recovered from the coronary arteries (Hruban *et al.*, 1990).

Clinical evidence indicating that CMV infection enhances the development of cardiac allograft vasculopathy is further supported by studies in animal models (reviewed in Koskinen *et al.*, 1999). In experimental animal models of rat transplanted with aortic or cardiac allografts, rat CMV (RCMV) infection resulted in an early inflammatory response that leads to enhanced intimal thickening in the allograft vascular wall (Lemstrom *et al.*, 1993; Koskinen *et al.*, 1994). In a study by Lemstrom *et al.* (1995), RCMV infection accelerated cardiac allograft atherosclerosis, particularly in small intramyocardial arterioles. IHC revealed upregulated presence of helper T cells, CTL, NK cells and macrophages in the perivascular space of arterioles; this imflammatory infiltrate also showed increased MHC class II molecule expression. RCMV early and late Ags were demonstrated in both vascular wall smooth muscle cells and macrophages, and replicating virus was present in allograft structures (Lemstrom *et al.*, 1995). This enhanced intimal response in aortic and cardiac allografts can be entirely abolished by the administration of prophylactic GCV, a potent inhibitor of CMV replication (Soghikian *et al.*, 1996; Lemstrom *et al.*, 1997), thus implicating CMV as a causative agent in chronic rejection.

1.3.9.3 Solid organ allograft rejection

In renal transplant recipients HCMV infection has been linked with acute allograft rejection, which in turn may lead to graft loss (von Willebrand *et al.*, 1986; Pouteil-Noble *et al.*, 1993; Sagedal *et al.*, 2002). In a study by Sagedal *et al.* (2002), HCMV disease was found to constitute an independent risk factor for biopsy-proven acute tubulointerstitial but not vascular rejection in renal transplant recipients. In a study of 192 consecutive renal transplant recipients, HCMV disease but not symptomless viraemia was seen as a risk factor for acute rejection (Toupance *et al.*, 2000). Support for the role of HCMV in triggering acute allograft rejection can be found in a study where administration of GCV, without conventional antirejection therapy resulted in stable improved graft function in renal transplant patients with HCMV infection and biopsy proven late acute rejection (Reinke *et al.*, 1994). Similarly, in a randomized, double-bind, placebo-controlled trial by Lowance *et al.* (1999), in which VACV was reported to prevent HCMV disease after renal transplantation (the incidence of HCMV disease ninety days after transplantation was 45% among placebo-treated patients and only 3% among VACV-treated patients; at 6 months, the incidence was 45% and 16% respectively), a reduction of biopsy confirmed acute rejection was observed in seronegative VACV recipients compared with placebo recipients (52% versus 26%) at six months after transplantation. These findings suggested that HCMV infection contributes significantly to acute graft rejection in the kidney.

The role of HCMV in the pathogenesis of CAN remains controversial. HCMV disease has been found to be associated with both early graft dysfunction and chronic allograft nephropathy (Tong *et al.*, 2002). One recent study found that HCMV disease only increased the risk for chronic rejection in renal patients who have experienced serious episodes of acute rejection (Humar *et al.*, 1999). Other studies have not found an association between HCMV and chronic rejection in the kidney (Nadasdy *et al.*, 1994). In a recent study, the HCMV inclusions in renal allograft biopsies were not associated with inflammatory response, and graft outcome was determined by coexisting acute rejection and CAN rather than CMV infection (Kashyap *et al.*, 1999).

Several rat models of chronic renal allograft rejection have also shown that RCMV infection increases the inflammatory response and accelerates chronic rejection (Yilmaz *et al.*, 1996; Lautenschlager *et al.*, 1997b). In the study by Kloover *et al.* (2000), RCMV caused a significant prolonged increase in vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in the vascular endothelium, and the number of cells expressing activation markers, leukocyte function antigen-1 (LFA-1) and very late antigen-4 (VLA-4) was significantly enhanced in these animals. Significantly enhanced histological changes of chronic rejection were also seen in the RCMV-infected rats. Recently, a rat kidney transplant model also showed RCMV increased collagen synthesis and the generation of fibrosis during the development of CAN (Inkinen *et al.*, 2002).

In liver transplantation, HCMV infection causes severe hepatitis and systemic infection in liver graft recipients (Stratta *et al.*, 1989). HCMV hepatitis has been found to be a rare event with an incidence of 2.1% in a series of 1146 liver transplantations (Seehofer *et al.*, 2002). A study focusing on HCMV hepatitis showed an increase incidence of chronic rejection in those patients (Manez *et al.*, 1993). However, these findings have not been confirmed in a recent study where long-term complications of HCMV hepatitis were not observed, and no correlation with chronic rejection was found (Seehofer *et al.*, 2002).

Several studies have also suggest that HCMV infection maybe a risk factor in the evolution of VBDS, a chronic cholestatic disorder, characteristic of chronic rejection (O'Grady *et al.*, 1988; Arnold *et al.*, 1992; Lautenschlager *et al.*, 1997a; Evans *et al.*, 2000). The persistence of HCMV DNA has been demonstrated in liver hepatocytes (Arnold *et al.*, 1992; Evans *et al.*, 1999) and in bile ducts and vascular structures (Lautenschlager *et al.*, 1997a) of liver grafts with chronic rejection. These findings, however, have not been confirmed in a prospective study of 81 consecutive liver transplant recipients where the occurrence of HCMV infection did not correlate with VBDS in the allograft recipients (Paya *et al.*, 1992). A recent study also showed that neither HCMV viraemia nor HCMV disease after liver transplantation had an adverse affect on the incidence and grade of acute rejection episodes during the first year after grafting in patients with HCV-related cirrhosis (Teixeira *et al.*, 2000). On the other hand, a recent study investigating bile duct complications during 1 year after liver

transplantation found a clear association between HCMV infection and biliary complication rate (Halme *et al.*, 2003). HCMV Ags and DNA were also demonstrated in the bile ducts.

More recently, support of CMV in hepatic allograft rejection has been reported in rat liver allograft models. In liver allografts undergoing acute rejection, RCMV significantly increased portal inflammation and caused more severe bile duct damage linked to the induction of VCAM-1 in the endothelial cells (Martelius *et al.*, 1998). A further study showed vascular adhesion protein-1 (VAP-1), an adhesion molecule involved in lymphocyte adhesion, was upregulated in acute liver rejection of sinusoids, hepatocytes in bile duct, and this upregulation was prolonged by RCMV. Thus, the severity of acute rejection was intensified (Martelius *et al.*, 2000).

1.3.10 Bidirectional effects of HCMV with allograft rejection

The exact nature of the interaction between allograft dysfunction and rejection, and HCMV infection is controversial, in the absence of specific pathological patterns directly attributable to HCMV. The relation between rejection and HCMV appears to be bi-directional, with acute HCMV infection accelerating rejection, and inflammation caused by rejection may increase viral replication. Thus, allograft rejection is associated with HCMV, although it may not be exclusively caused by HCMV.

1.3.10.1 Effects of allograft rejection on HCMV infection

There are two key steps in the pathogenesis of HCMV infection in allograft recipients; reactivation of the virus from latency and viral replication and dissemination (reviewed by Tolkoff-Rubin *et al.*, 2001; Rubin, 2001). The effects of the rejection process on the pathogenesis of HCMV include those that stem from the rejection process itself, and those due to immunosuppressive drugs required to prevent and treat rejection.

A potential mediator involved in reactivating HCMV from latency is the proinflammatory cytokine, TNF-α. This has been demonstrated in an animal model where allogeneic transplantation was found to induce CMV IE-1 gene expression and the expression of cytokines such as TNF (Hummel and Abecassis, 2002). TNF-α associates with the TNF-receptor on latently infected cells, resulting in activation of protein kinase C and NF_kB, a promoter of the IE gene of HCMV that initiates viral

replication (Stein *et al.*, 1993; Prosch *et al.*, 1995). Allograft rejection results in both local and systemic TNF release, and therefore the amount of TNF released will determine the amount of viral reactivation. Two other pathways have been shown to lead to HCMV reactivation (reviewed in Reinke *et al.*, 1999). They include the stress mediators, epinephrine and norepinephrine, which activate HCMV by increasing cyclic AMP (cAMP) resulting in IE enhancer/promoter stimulation as well as viral replication *in vitro*. Proinflammatory prostaglandins can also contribute to HCMV reactivation via the same cAMP pathway. Hence, common events that occur during allograft rejection such as TNF release, stress, and inflammation can mediate the critical first step in the pathogenesis of HCMV infection (see figure 1.4).

The type and intensity of immunosuppressive therapy for the management of rejection also contributes significantly to viral replication and disease. The administration of ATG, OKT3 or other antilymphocyte Abs to control rejection results in release of large amounts of TNF and other proinflammatory factors that are associated with the activation of the virus (Hibberd *et al.*, 1992; 1995; Turgeon *et al.*, 1998). Drugs such as calcineurin inhibitors, cyclosporine and tacrolimus do not reactivate HCMV from latency, but once there is replicating virus, the effects of HCMV will be amplified through inhibition of virus specific, cytotoxic T-cell response. A similar effect is demonstrated by high-dose corticosteroids.

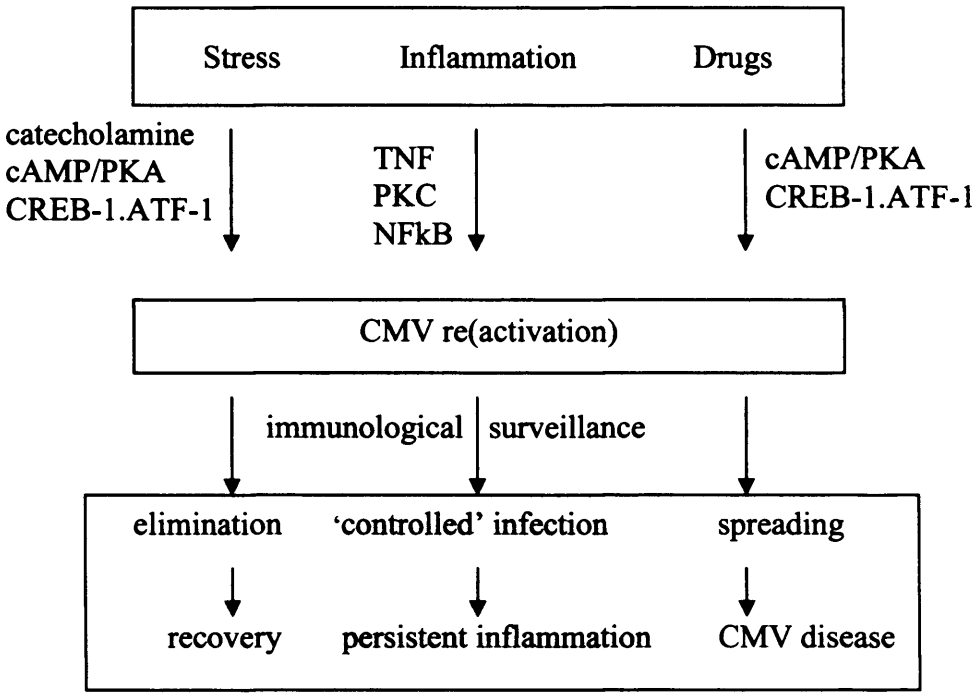


Figure 1.4 HCMV is (re)activated by very 'ordinary events' and the immune response determines the outcome of an active infection (Reinke, 1999). Different events activate HCMV via distinct intracellular pathways. The immune response determines the clinical outcome: normally the virus is rapidly eliminated by cellular immunity while in severely immunocompromised patients the virus can replicate and spread without control resulting in 'classic' HCMV disease. A partially deficient immune response may prevent uncontrolled spreading and replication but insufficient clearance resulting in persistent virus mediated inflammatory processes.

1.3.10.2 Effects of HCMV infection on allograft rejection

A number of potential mechanisms have been proposed to account for the effects of HCMV infection on the allograft. A direct form of injury has also been suggested. Sequence homology and immunologic crossreactivity between an IE-2 protein of HCMV and the HLA-DR β-chain have been demonstrated (Fujinami *et al.*, 1988). In addition, the HCMV UL18 gene encodes a protein with homology to the class I heavy chain (Beck and Barrell, 1988), which can complex with the light chain (β-2-microglobulin) (Browne *et al.*, 1990) and is able to bind peptides similar to HLA class I (Fahnestock *et al.*, 1995). Thus, immune injury triggered by the virus could be directed at cells that bear either the appropriate HLA-DR Ag or the particular class I Ag in a form of molecular mimicry (Fujinami *et al.*, 1988; Beck and Barrell, 1988). In a recent study by Saverino *et al.* (2004) HCMV UL18 was detected in organ biopsies from immunocompromised patients with productive infection. HCMV UL18 was found to bind to T cells via CD85j, in the absence of CD3/TCR involvement, resulting in activation of T cells and the subsequent lysis of HCMV infected cells. Pietra *et al.* (2003) also showed that HCMV UL40 peptides presented by HLA-E can stimulate some CD8+ T cells in addition to inhibiting NK cell killing (Wang *et al.*, 2002).

As mentioned previously, allograft rejection has been associated with the increased expression of intercellular adhesion molecules. It has been shown that HCMV infection in fibroblasts causes an increased expression of ICAM-1 (CD54) and LFA-3 (CD58) (Craigen and Grundy, 1996; Grundy and Downes, 1993). *In vivo*, HCMV was shown to infect endothelial cells, causing an increase in the expression of the adhesion molecules ICAM-1 and VCAM-1, which was accompanied by an increase in the infiltration of inflammatory cells into the allograft, particularly CD11a (LFA-1) and CD49d (VLA-4)-positive cells (Steinhoff *et al.*, 1996). Allograft rejection is also associated with the upregulation of MHC Ags on allograft cells, which may in part be due to the expression of IFN. In human renal transplant recipients, an increased HLA class II Ag expression was seen in the graft during active HCMV infection, and such increase was associated with acute rejection (von Willebrand *et al.*, 1986). CMV infection may directly increase MHC Ags on the surface of graft cells through the induction of proinflammatory cytokines as such as TNF-α and IFN-γ (reviewed in Cainelli and Vento, 2002) and may activate cytotoxic T cells, which can trigger acute rejection in association with concurrent alloantigen stimulation. In the murine CMV model the cytotoxic T-cell

response to alloantigens has been shown to be increased during CMV infection *in vivo* (Grundy and Shearer, 1984). Kas-Deelen *et al.* (2000) have postulated that the occurrence of acute rejection episodes at the allograft site sensitizes the endothelial surface of the host to HCMV-induced damage. These endothelial cells then become a target for alloreactive T cells.

HCMV infection of allograft recipients has also been shown to induce a polyclonal activation of humoral immune responses, including an 80% incidence of anti-endothelial cell Abs (AECAs) that could contribute to allograft injury (Toyoda *et al.*, 1999).

1.3.11 Prevention and treatment of HCMV infection and disease

HCMV disease is a major problem after organ transplantation and in individuals infected with HIV. Consequently, antiviral agents have been introduced in clinical practice to inhibit HCMV replication. The currently approved drugs for use against HCMV include GCV, foscarnet (PFA) and cidofovir (CDV). In addition to the treatment of overt clinical disease two major strategies are employed: prophylaxis with antiviral agents and pre-emptive therapy for the management of HCMV infection.

1.3.11.1 Antiviral Chemotherapy

To date, GCV, CDV and PFA have been licensed for serious or life-threatening HCMV infections in immunocompromised individuals. These drugs have produced clinical improvement in many patients, but suffer from poor oral bioavailability, low potency, development of drug resistance, and dose-limiting toxicities, and hospitalization is sometimes required.

1.3.11.1.1 Ganciclovir (GCV)

Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)-guanine] is a guanosine nucleoside analogue that requires phosphorylation at the 5' position for antiviral activity. The initial phosphorylation in HCMV infected cells is mediated by the HCMV encoded UL97 gene product (Littler *et al.*, 1992). The modified drug is then converted into GCV triphosphate through the action of cellular enzymes. The fully activated GCV triphosphate inhibits viral replication by competing with deoxyguanosine triphosphate (dGTP) as a substrate for the viral DNA polymerase (HCMV UL54) (Noble and Faulds,

1998). The incorporation of GCV triphosphate into the growing chain of viral DNA does not lead to obligatory chain termination but rather slows the rate of DNA polymerization. GCV is administered by intravenous infusion, although more recently an oral formulation has been used. While this has proved effective in preventing HCMV disease in SOT recipients (Gane *et al.*, 1997; Flechner *et al.*, 1998), it has a low bioavailability (about 6-10%). This low bioavailability limits the degree of viral suppression and thus may predispose to the emergence of GCV resistant strains (discussed in section 1.3.11.2).

1.3.11.1.2 Foscarnet (PFA)

Foscarnet (phosphono formic acid; PFA) is a pyrophosphate analogue which binds directly to the pyrophosphate-binding site of the viral DNA polymerase in a non-competitive manner resulting in the inhibition of DNA polymerization. PFA is not used routinely in transplant patients because of its nephrotoxicity. In general, it is reserved for patients who are intolerant of GCV, or who have failed GCV therapy, usually due to resistance. However, in a recent study by Mattes *et al.* (2004) comparing GCV to GCV plus PFA (each at half dose) for pre-emptive therapy of HCMV infection in transplant recipients, a synergistic effect of GCV plus PFA *in vivo* was not supported.

1.3.11.1.3 Cidofovir (CDV)

Cidofovir (HPMPC, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine) is an acyclic nucleotide phosphonate, an analogue of cytosine monophosphate and therefore does not require virally mediated initial phosphorylation (Kendle and Fanhavard, 1998). CDV is then converted by cellular enzymes to a triphosphate form which inhibits viral DNA synthesis. The main side effect of cidofovir is its nephrotoxicity, which can be accompanied with neutropenia. CDV is mainly used to treat HCMV infections in patients who have acquired resistance to either GCV or PFA.

1.3.11.2 Prophylaxis

Prophylaxis is where an antiviral is administered to all patients after transplantation in order to prevent HCMV infection/disease from occurring. At present, prophylaxis against HCMV has used drugs such as aciclovir (ACV) or its prodrug VACV or oral GCV. Clinical trials of these agents in SOT have been extremely successful at controlling HCMV replication for the defined period of prophylaxis (Gane *et al.*, 1997; Lowance *et al.*, 1999). In the study by Lowance *et al.* (1999) prophylactic use of oral VACV for ninety days post-transplant reduced the incidence of HCMV disease in the D+/R- group from 45% (placebo) to 3% (VACV), and reduced the rate of biopsy-confirmed acute graft rejection at 6 months from 52% (placebo) to 26% (VACV). However, at six months post-transplant, the incidence of disease was the same in the placebo group, but had increased to 16% in the VACV group.

It has become increasingly evident that approximately 25-30 days following the end of antiviral prophylaxis, late-onset HCMV infection and disease has become a problem (Emery, 2001). This is intensified by the delay in antiviral drug intervention when viral loads are low due to infrequent outpatient visits, thus resulting in an increased risk of HCMV disease and viral replication that is more difficult to control. Furthermore, since the duration of therapy required to reduce viral load to undetectable levels is directly related to the initial viral load, these patients will require an extended period of intravenous (iv) GCV therapy. Another potential problem concerning prophylactic therapy is the development of resistance of HCMV to the antiviral drugs. Mutations in the UL97 protein kinase, the UL54 CMV DNA polymerase or both are the major mechanisms by which HCMV becomes resistance to GCV (Chou *et al.*, 1999). Resistance to GCV following prophylaxis has been reported in organ transplant recipients (Limaye *et al.*, 2000). Cross-resistance to other antiviral agents has also been demonstrated (Baldanti *et al.*, 1996). Recently, valganciclovir (VGCV), a valine ester prodrug of GCV, was developed to overcome the limitations of GCV. This drug is currently undergoing clinical trials, and there is limited data available on VGCV prophylaxis in organ transplant recipients. In one recent study, oral VGCV (900 mg once-daily) was as clinically effective and well-tolerated as oral GCV (1000 mg three times a day) for HCMV prevention in D+R- SOT recipients. The safety profile was also similar for both drugs (Paya *et al.*, 2004).

1.3.11.3 Pre-emptive therapy

Pre-emptive therapy is given to high-risk patients on the basis of detection of HCMV in the blood (Emery, 2001). Since the drug is only targeted to patients who are at risk of future HCMV disease, it reduces the number of patients exposed to the potentially toxic antiviral drug, lowers the risk of drug resistance, and maximizes cost-benefit. The success of pre-emptive therapy is dependent on early diagnosis of HCMV infection using sensitive detection methods such as PCR, nucleic acid sequence-based amplification (NASBA) and antigenemia. In our centre, pre-emptive therapy is initiated when two consecutive blood samples are HCMV DNA positive by PCR (Mattes *et al.*, 2004). Studies in BMT and SOT recipients have shown that using either PCR or antigenemia to initiate pre-emptive therapy is superior to therapy initiated on the basis of cell culture results (Hebart *et al.*, 1997; Boeckh *et al.*, 1996). A number of studies involving the pre-emptive treatment of high risk transplant patients have demonstrated a reduction in the rate of HCMV disease comparable to that of universal prophylaxis (Boeckh *et al.*, 1996; Kusne *et al.*, 1999).

1.4 Human herpesvirus 6 (HHV-6)

1.4.1 Discovery

HHV-6 was first isolated in 1986 from the PBMCs of six patients with lymphoproliferative disorders, some of whom were infected with HIV-1 (Salahuddin *et al.*, 1986). The herpesvirus was originally designated human B-lymphotropic virus (HBLV) based on its apparent *in vitro* tropism for freshly isolated B-cells. However, subsequent laboratories established that this newly described virus was most likely to be found and to grow in CD4+ T lymphocytes (Downing *et al.*, 1987; Lopez *et al.*, 1988; Lusso *et al.*, 1988; Takahashi *et al.*, 1989). The virus was subsequently renamed HHV-6, in accordance with the guidelines established by the ICTV (Ablashi *et al.*, 1987). The HHV-6 genome is 160-162 kbp, and contains over 100 ORFs. More than 65% of the HHV-6 predicted genes have a recognized counterpart in HCMV (Gompels *et al.*, 1995).

1.4.2 HHV-6 variants A and B

HHV-6 consists of two closely related yet distinct viruses, designated HHV-6A and HHV-6B. Recently, the complete nucleotide sequence of HHV-6A prototype strain U1102 and HHV-6B strains Z29 and HST has been determined (Gompels *et al.*, 1995; Isegawa *et al.*, 1999). HHV-6A and HHV-6B have an overall nucleotide sequence identity of 90%. However, the two variants differ with respect to a number of properties including restriction enzyme profiles, nucleotide sequences, growth properties *in vitro* and reactivity with panels of monoclonal Abs (mAbs) (Ablashi *et al.*, 1991; Aubin *et al.*, 1993).

1.4.3 Propagation and Cell Tropism

The primary target cells for HHV-6 are CD4+ T-lymphocytes (Lusso *et al.*, 1988; Takahashi *et al.*, 1989). All HHV-6 isolates replicate in activated peripheral or cord blood mononuclear cells, but can be adapted to grow in continuous T-cell lines that include HSB-2 and JJHAN cells for HHV-6A, and Molt-3 and MT-4 T cells for HHV-6B (Ablashi *et al.*, 1991). Although HHV-6 most efficiently replicates in activated T cells, it has the ability to infect a broad range of cell types *in vitro* including lymphocytes, monocytes, epithelial cells, endothelial cells, fibroblasts, glioblastoma cells, fetal astrocytes and adult oligodendrocytes (Ablashi *et al.*, 1987; Kondo *et al.*, 1991; He *et al.*, 1996; Albright *et al.*, 1998; Wu and Shanley, 1998). *In vivo*, CD4+ T

cells and monocytes appear to be the preferential target for fully permissive replication (Takahashi *et al.*, 1989; Kondo *et al.*, 2002b). In addition, CD8+, gamma/delta (γδ) T-lymphocytes, and NK cells support HHV-6 replication in association with surface expression of CD4 (Lusso *et al.*, 1993; 1995). An HHV-6A and HHV-6B cellular receptor (CD46) was recently identified (Santoro *et al.*, 1999). CD46, a ubiquitous type-1 glycoprotein (also a cellular receptor for vaccine strains of measles virus and group B adenovirus), is expressed on the surface of all nucleated cells and is a regulator of complement activation (Santoro *et al.*, 1999).

1.4.4 Epidemiology and virus transmission

Seroepidemiologic studies have shown that HHV-6 is ubiquitous in the population, seroprevalence is over 90% in healthy adults, and infection is widespread throughout the world. The majority of individuals undergo primary HHV-6 infection during childhood, usually within the first two years of life (Okuno *et al.*, 1989). Viral loads in blood are also high during primary infection and appear to peak at around 10^5 genomes/μg PBMC DNA (Clark *et al.*, 1997). The most likely route of transmission of HHV-6 is the saliva. HHV-6 DNA has been detected in saliva of children and healthy adults by PCR, and there is evidence of horizontal infection through saliva within families (van Loon *et al.*, 1995). HHV-6B DNA has been detected by PCR in the cervices of nearly 20% of 72 women in the late stages of their pregnancies, compared with 6% of non-pregnant controls (Okuno *et al.*, 1995), and in the female genital tract by PCR (Leach *et al.*, 1994) suggesting perinatal virus transmission through transvaginal delivery. Intrauterine transmission of HHV-6 has also been reported. Viral DNA is detectable in fetus or blood of newborn babies (Aubin *et al.*, 1992; Hall *et al.*, 1994).

Most HHV-6 infections in children are believed to be due to HHV-6B. HHV-6B was detected by PCR in oropharyngeal secretions and PBMCs of >90% of children, whereas HHV-6A was found in <3% (Hall *et al.*, 1998). However, the true prevalence of HHV-6A appears to be underestimated by testing blood and saliva (Clark, 2003). HHV-6A is more frequently detected in skin biopsies (Di Luca *et al.*, 1996) and cerebral spinal fluid (CSF) (Hall *et al.*, 1998).

1.4.5 Primary infection

Primary infection with HHV-6B has been shown to be associated with exanthem subitum (ES) (roseola infantum), a common, mild acute febrile disease of infants (Yamanashi *et al.*, 1988). Although febrile illness is usually self-limiting, it accounted for nearly 10% of visits of children with acute febrile illness to a hospital emergency room (Hall *et al.*, 1994). Complications of primary HHV-6 infections have also been reported, but are uncommon. The most common is febrile convulsion and more rarely meningitis (Huang *et al.*, 1991), hepatitis (Asano *et al.*, 1990), meningoencephalitis (Ishiguro *et al.*, 1990) and fatal encephalitis (Asano *et al.*, 1992). Reports of HHV-6 DNA detection in CSF of children complicated by seizures (Kondo *et al.*, 1993; Suga *et al.*, 1993) indicate that neurological complications in children infected with HHV-6 are a likely result of HHV-6 directly infecting the central nervous system (CNS). Primary infection in adults is relatively rare and more severe than in children. An EBV-negative infectious mononucleosis (Steeper *et al.*, 1990), prolonged lymphadenopathy (Niederman *et al.*, 1988), and fulminant hepatitis (Sobue *et al.*, 1991) have been described.

1.4.6 HHV-6 persistence in the host

Like the other human herpesviruses, HHV-6 persistently infects humans after primary infection. This persistence is likely to include both a latent state with infectious virus only produced during episodes of reactivation, and chronic replication with production of infectious virus. Salivary glands are a candidate site of chronic infection as HHV-6 is frequently detected in the saliva by PCR (Jarrett *et al.*, 1990). In a study by Sada *et al.* (1996) HHV-6 DNA was demonstrated in three different salivary glands (submandibular, parotid and lip), although the submandibular gland (88%) had the highest frequency of HHV-6 DNA compared to 63% in the parotid gland and 53% in the lip salivary gland.

HHV-6 can be detected in the PBMCs using nested PCR, reflecting low viral loads (Clark *et al.*, 1996) and most likely latent virus. The detection of HHV-6 U94 mRNA in the peripheral blood of healthy individuals has been reported suggesting latency-associated gene expression (Rotola *et al.*, 1998). The actual site of HHV-6 latent infection is not completely clear, although possible sites include monocytes and early bone marrow progenitor cells (Kondo *et al.*, 1991; Luppi *et al.*, 1999). HHV-6A and

HHV-6B are also neurotropic. In a study investigating the presence of HHV-6 in post mortem brain tissue, up to 85% of individuals harboured HHV-6 DNA in the brain as detected by PCR. HHV-6B was predominantly detected, although HHV-6A was detected in 27.5% of brains and co-infections were also observed (Chan *et al.*, 2001). These results suggest that human brain may be another site for HHV-6 latency. HHV-6B has been shown to be reactivated from latency by infection with HHV-7 but not by T-cell stimulation *in vitro* (Katsafanas *et al.*, 1996). The stimuli for HHV-6 reactivation *in vivo* are uncharacterized, but are likely to include immunosuppression.

Some studies have also demonstrated widespread distribution of HHV-6 DNA in organs derived from both immunocompetent and immunocompromised hosts (Clark *et al.*, 1996; Emery *et al.*, 1999b) with overall low viral loads detected in the majority of tissues. In the study by Clark *et al.* (1996), HHV-6 viral loads were however, significantly increased in autopsy tissues from AIDS patients compared to controls suggesting upregulation of HHV-6 replication in the former.

1.4.7 HHV-6 integration

An alternative form of HHV-6 persistence characterized by very high viral loads in PBMC and integration of apparently whole-genome length HHV-6 sequences into host-cell chromosomes is present in a small but significant subset of the population (Luppi *et al.*, 1993; Torelli *et al.*, 1995). Integration of HHV-6 into host cell chromosomes was first reported in three individuals (B cell lymphoma, Hodgkin's disease, and a patient with multiple sclerosis) who had unusually high copy numbers of HHV-6 in their PBMC (Luppi *et al.*, 1993). Abnormally high viral loads found in PBMC may represent integration in a bone marrow progenitor cell which gives rise to a range of differentiated cells, each harbouring viral sequences (Luppi *et al.*, 1999). Further analysis by fluorescence *in situ* hybridisation (FISH) with HHV-6 specific probes showed in all three cases the presence of a specific hybridisation site, located on the short arm of chromosome 17 (Torelli *et al.*, 1995). Morris *et al.* (1999) suggested that the sites of integration were close to or in the telomeres of chromosome 17 (Morris *et al.*, 1999). At present the exact molecular mechanisms of HHV-6 integration to chromosomal sites are unknown. There are human telomeric-like repeat sequences at both ends of the HHV-6 genome (Gompels and Macaulay, 1995), which may be relevant to the mode by which the virus integrates into human chromosomal DNA (Clark, 2000; 2003).

Recently, the possibility of chromosomal transmission of HHV-6 DNA has been reported (Daibata *et al.*, 1999). A woman with Burkitts lymphoma, and her asymptomatic husband had HHV-6 DNA integrated at chromosome 22q13 and 1q44 respectively. Their daughter was found to have integrated virus at both these chromosomal sites suggesting entry of viral sequences into the germline and chromosomal inheritance (Daibata *et al.*, 1999).

The clinical significance of integrated forms of HHV-6 sequences remains uncertain, although viral reactivation from an integrated latent state has been demonstrated for Marek's disease virus, which is a T-cell tropic herpesvirus that infects birds (Delecluse *et al.*, 1993). Integrated virus is also a potential confounder in studies investigating HHV-6 disease associations and also in the medical management of infection (Emery and Clark, in press). Unproven associations between HHV-6 and disease may reflect the unrecognized detection of integrated virus and interpreted as a high viral load. From the management perspective, it is important to differentiate patients with active HHV-6 infection from those patients with integrated virus to prevent the latter receiving unnecessary exposure to potentially toxic antiviral drugs.

1.4.8 HHV-6 and immunomodulation

Based on *in vitro* studies, HHV-6 has potential immunomodulating properties including the ability to alter the expression of immune activation molecules (reviewed by Clark, 2003).

Productive infection of HHV-6 has a variety of effects on T cells. The virus downregulates CD3 in infected T cells (Lusso *et al.*, 1991). Because the CD3/TCR complex is important in the T cell activation processes, downregulation of CD3 may have an immunosuppressive effect. HHV-6 has also been shown to upregulate the chemokine receptor, CCR7, in infected T cells (Yoshida *et al.*, 1997) and to down-regulate CXCR4 expression by decreasing the association of the CXCR4 gene repressor, YY1, thereby altering the chemotactic response of directly infected cells (Hasegawa *et al.*, 2001). Downregulation of CD46 also occurred following HHV-6 infection (Santoro *et al.*, 1999). In addition, HHV-6A has been reported to downregulate HLA class 1 molecules in infected dendritic cells, suggesting a potential immune evasion strategy (Hirata *et al.*, 2001). HHV-6 may also modulate the

expression of a number of cytokines. HHV-6 is potent inducer of IFN-γ, TNF-α, and IL-1β (Flamand *et al.*, 1991) as well as IL-10 and IL-12 expression in monocytes/macrophages (Li *et al.*, 1997). HHV-6 infection of PBMC was reported to upregulate NK cell cytotoxicity, which may be mediated in part by increased expression of IL-15 (Flamand *et al.*, 1996). Furthermore, infection of PBMC by HHV-6 has been found to suppress T cell function including reduced IL-2 synthesis and cell proliferation (Flamand *et al.*, 1995).

HHV-6 also possess genes with homology to the GPCRs (U12 and U51). HHV-6 U12 functionally encoded a calcium-mobilizing receptor for β-chemokines RANTES, MIP-1 α and -1β, and MCP-1, but not the α-chemokine IL-8 (Isegawa *et al.*, 1998). The product of U12 may therefore play an important role in the pathogenesis of HHV-6 through transmembrane signaling. HHV-6 U51 showed specific binding of the CC chemokine RANTES, and in epithelial cells already secreting RANTES, U51 expression led to specific transcriptional downregulation (Milne *et al.*, 2000).

HHV-6 also encodes a potential viral chemokine. The HHV-6B U83 protein was found to be capable of inducing transient calcium mobilization and to chemotactically activate human monocytic THP-1 cells (Zou *et al.*, 1999). Recently, the chemokine receptor CCR2 which binds U83B has been reported (Luttichau *et al.*, 2003). These findings suggest that the protein may play a critical role in HHV-6 replication *in vivo* by activating and recruiting mononuclear cells. However, the U83 gene in HHV-6A strains appears to lack a signal peptide and may therefore not be secreted from an infected cell (Zou *et al.*, 1999).

1.4.9 HHV-6 infection following transplantation

Like other members of the herpesviruses, HHV-6 has the potential for enhanced pathogenicity in the immunocompromised host such as those iatrogenically-suppressed as a result of transplantation. Studies using both cell culture and more sensitive PCR-based assays have shown that infection with HHV-6 in the post-transplant period is common. The peak incidence of HHV-6 infection is 2-4 weeks post-transplantation (Griffiths *et al.*, 2000) and although the source of the virus has not been precisely defined, the high HHV-6 rate of seropositivity in the general population suggests most infections in transplant patients are likely to result from reactivation of the recipient's

latent virus. This has been demonstrated to occur in a case where a transplant recipient had two HHV-6 strains isolated before and after BMT. Restriction fragment length polymorphism analysis of both strains indicated that the virus which later reactivated was the same as that harboured latently in the recipient (Yoshikawa *et al.*, 1991). However, HHV-6 DNA has been detected in 28% of bone marrow samples from healthy individuals by PCR indicating a possible source of virus transmission from the donor to the recipient (Gautheret-Dejean *et al.*, 2000), and cases of primary infection have been reported in BMT recipients (Lau *et al.*, 1998), and in living-related liver transplantation (Yoshikawa *et al.*, 2001). Both HHV-6A and HHV-6B have been detected in the post-transplant period, although the latter is more common.

1.4.9.1 HHV-6 infection in bone marrow transplant recipients

Most studies delineating the potential pathogenic role of HHV-6 in transplant patients have arisen from BMT recipients. The reported incidence of HHV-6 infection following BMT ranges from 28% to 75% (median 48%) depending on the diagnostic method (Dockrell and Paya, 2001).

Case reports and retrospective studies have reported the association of HHV-6 with a range of disease manifestations including pneumonitis (Carrigan *et al.*, 1991; Cone *et al.*, 1993), bone marrow suppression (Drobyski *et al.*, 1993; Carrigan and Knox, 1994), early and late graft failure (Rosenfeld *et al.*, 1995; Johnston *et al.*, 1999), and encephalitis (Drobyski *et al.*, 1994). A study of 16 BMT patients from which HHV-6 was directly isolated from the blood of 6 patients, showed idiopathic marrow suppression occurred more frequently in 67% (4 of 6) of patients with concurrent HHV-6 viraemia, than in 10% (1of 10) of patients without viraemia ($P<0.05$). In addition, HHV-6 was cultured from the bone marrow of all four patients with viraemia at the time of marrow suppression (Drobyski *et al.,* 1993). This is supported by *in vitro* evidence of HHV-6 suppression on the maturation and growth of normal bone marrow precursors, including granulocyte/macrophage, erythroid, and megakaryocytic lineages (Burd *et al.*, 1993; Drobyski *et al.*, 1993; Isomura *et al.*, 1997; 2000). To critically assess the clinical impact of HHV-6 following BMT, a number of prospective studies have been carried out (see table 1.8). In some of these studies where the whole population was analysed, definitive HHV-6 associated diseases have not been established. However, subgroup analysis has reported the association of HHV-6 with clinical illness including rash, delayed engraftment, and graft versus host disease

(GVHD) (Cone *et al.*, 1999; Yoshikawa *et al.*, 1991; Wilborn *et al.*, 1994; Appleton *et al.*, 1995). Other studies have not linked HHV-6 with clinical disease (Kadakia *et al.*, 1996; Chan *et al.*, 1997).

Study	Method of Detection	No. of Pts	No. of bloods	Observed disease
Wilborn et al (1994)	PCR	57	514	GVHD*
Appleton et al (1995)	PCR	57	NG	GVHD* (PCR skin/rectal biopsies)
Kadakia et al (1996)	VI	26	NG	None
Wang et al (1996)	PCR	37	270	Delayed granulocyte and platelet engraftment*
Chan et al (1997)	PCR	61	563	None
Cone et al (1999)	PCR	20	264	Rash*
Maeda et al (1999)	PCR	38	209	Delayed platelet engraftment
Ljungman et al (2000)	QCPCR	74	494	Viral load correlated with delayed platelet engraftment
Imbert-Marcille et al (2000)	PCR	92	961	Myelosuppression and fever
Yoshikawa et al (2002)	VI	82	NG	Rash*

Table 1.8 Prospective studies of HHV-6 post bone marrow/ stem cell transplantation (Clark, 2003).

Abbreviations: * analysis on subgroup of patient population; NG, not given; PCR, polymerase chain reaction; VI, virus isolation

1.4.9.2 HHV-6 infection in solid organ transplant recipients

As with BMT, HHV-6 infection occurs frequently after SOT with detection rates varying from 0% to 82% (median 32%) again depending on the assay used (Dockrell and Paya, 2001). The clinical impact of HHV-6 infection after liver transplantation has been mostly supported by case reports where HHV-6 associated bone marrow suppression, interstitial pneumonitis, encephalitis and hepatitis (Singh *et al.*, 1997; Singh and Paterson, 2000; Ward *et al.*, 1989) have been reported. However, several prospective studies examining the pathogenic role of HHV-6 in patients who have undergone liver or renal transplantation have failed to show any particular disease association (Schmidt *et al.*, 1996; Herbein *et al.*, 1996; Osman *et al.*, 1996; Kidd *et al.*, 2000) (see table 1.9).

Although prospective studies report a low incidence of HHV-6 end-organ disease, the indirect effects of this virus are likely to be significant in the post-transplant period (Humar *et al.*, 2002; Mendez *et al.*, 2001; Griffiths *et al.*, 1999a). As mentioned above, HHV-6 is common after transplantation, therefore infection with more than one human β-herpesvirus may occur and interaction among these viruses could exacerbate existing pathological processes. A study by Desjardin *et al.* (1998) in renal transplant recipients showed HHV-6 reactivation, documented by a 4-fold rise in Ab titre, was associated with an increased risk of HCMV disease after primary HCMV infection. Similarly, in a cohort of 247 liver transplant recipients, HHV-6 seroconversion was significantly associated with symptomatic HCMV infection in HCMV-positive recipients of transplants from HCMV-positive donors (Dockrell *et al.*, 1997). Studies in both liver and renal transplant patients have shown that concomitant infection with both HHV-6 and HCMV is associated with more severe clinical disease (Herbein *et al.*, 1996; Ratnamohan *et al.*, 1998). HHV-6 infection has also been suggested to predispose to bacterial and fungal superinfections, possibly due to its immunosuppressive effects. HHV-6 seronegativity before transplantation has been shown to be an independent predictor of invasive fungal infection during the first 90 days after transplantation (Dockrell *et al.*, 1999). In a study of 200 liver transplant patients Humar *et al.* (2002) showed that HHV-6 infection was significantly associated with the development of opportunistic infections (predefined viral, fungal and bacterial disease) and HCMV disease.

Study	Transplant type	Method of detection	No. of pts	No. of bloods	Observed disease
Schmidt (1996)	liver	PCR	46	287	None
Herbein (1996)	liver, renal	VI	32	NG	None
Osman (1996)	renal	PCR	56	NG	None
Ratnamohan (1998)	renal, pancreas	PCR	30	NG	Fever
Griffiths (1999a)	liver	PCR	60	536	Graft rejection
Kidd (2000)	renal	PCR	52	596	None
Lautenschlager (2000)	liver	Ag	51	622	Graft dysfunction*
Rogers (2000)	liver	VI	80	NG	CNS disease Fungal infections
Mendez (2001)	liver	PCR	33	NG	CMV disease
Humar (2002)	liver	PCR	200	NG	Opportunistic infections CMV disease Graft rejection*

Table 1.9. Prospective studies of HHV-6 post solid organ transplantation (Clark, 2003).

Abbreviations: * analysis on subgroup of patient population; NG, not given; PCR, polymerase chain reaction; VI, virus isolation; Ag, antigenemia test

An association between HHV-6 and graft rejection has also been suggested. A study conducted in our centre (and which forms the basis of the work presented in chapter 5) of 60 consecutive liver transplant recipients showed that infections with HHV-6 and/or HHV-7 are common after transplantation, and that they occur earlier than HCMV infection (the median time to first PCR positive sample was 20 days for HHV-6, 26 days for HHV-7 and 36 days for HCMV) (Griffiths *et al.*, 1999a). The quantitative competitive PCR (QC-PCR) results also showed that the peak viral load of non-primary HCMV infection was significantly greater than that for HHV-6 and HHV-7. Clinicopathological analyses have shown that all three viruses were significantly associated with altered graft function, and that both HHV-6 and HCMV were independently associated with biopsy proven rejection suggesting that HHV-6 may be a previously unrecognized pathogen in this patient group (Griffiths *et al.*, 1999a). Consistent with these findings is a study by Lautenschlager *et al.* (2000) who used HHV-6 antigenemia assay for the diagnosis of HHV-6 infection. Of 51 consecutive liver transplant recipients, approximately 22% of patients had HHV-6 infection diagnosed during the first year after transplantation. Significant graft dysfunction consistent with organ rejection was associated with the detection of HHV-6 antigenemia in 8 of the 11 patients, and in 3 of these patients HHV-6 viral Ags could also be detected in the liver biopsy. HHV-6 could directly participate in the rejection process by triggering or exacerbating the inflammatory response. This is supported by the finding that HHV-6 infection in the liver increased the expression of adhesion molecules and the number of HLA class II positive cells (Lautenschlager *et al.*, 1999). Some studies have also linked HHV-6 infection with graft rejection in renal transplant patients (Okuno *et al.*, 1990; Jacobs *et al.*, 1994), although this has not been demonstrated by others (Merlino *et al.*, 1992).

1.5 Human herpesvirus 7 (HHV-7)

HHV-7 is the most recently discovered member of the β-herpesvirus subfamily of herpesviruses. HHV-7 is 145 kbp in genome size and potentially encodes at least 84 different proteins. HHV-7 is genetically most closely related to both HHV-6 variants, with nucleic acid sequence identities ranging from 20.7% to 75.7% in various genes (Dominguez *et al.*, 1996; Singer and Frenkel, 1997). The virus also shares some similarities in cell tropisms, disease associations, and some common antigenic epitopes (reviewed in Black and Pellett, 1999).

1.5.1 Discovery

HHV-7 was first isolated in 1990 from CD4+ T lymphocytes purified from PBMCs of a healthy HIV-negative donor. The cells were activated with anti-CD3 mAb together with either IL-2 or anti-CD28 mAb (Frenkel *et al.*, 1990). The cells exhibited a CPE after approximately 2 weeks in culture, and electron microscopic analysis revealed a characteristic herpesvirus structure. The virus could not be isolated from unstimulated cells. Using similar methods, additional strains of HHV-7 were isolated from peripheral blood lymphocytes (PBL) (Frenkel and Wyatt, 1992), and from the cell-free fraction of more than 75% of saliva specimens (Wyatt and Frenkel, 1992; Hidaka *et al.*, 1993) from healthy adults.

1.5.2 Propagation and Cell Tropism

HHV-7 has a narrow cell tropism of CD4+ T cells, such as phytohaemagglutinin (PHA)-stimulated human cord blood mononuclear cells (CBMCs) and PBMCs, after which infection can be transferred to the continuous CD4+ immature T-cell line SupT1 to support HHV-7 growth (Berneman *et al.*, 1992). HHV-7 uses CD4 as its cellular receptor for primary attachment onto susceptible cells (Lusso *et al.*, 1994), although CD4 alone is not sufficient to support a productive infection as suggested by the inability of HHV-7 to infect several CD4+ lymphoid cell lines (Yasukawa *et al.*, 1997). HHV-7 infection down-modulates cell surface CD4 expression (Furukawa *et al.*, 1994), as well as the CXCR4 in CD4 T cells (Secchiero *et al.*, 1998). Because of the competition for the CD4 receptor, *in vitro* HHV-7 is able to interfere negatively with HIV and to protect infected cells from HIV superinfection (Lusso *et al.*, 1994). *In vivo*, HHV-7 tropism is less restricted than in cell cultures. CD68-positive monocytes/macrophages appear to be infected with HHV-7 (Kempf *et al.*, 1997).

Zhang *et al.* (2001) have also demonstrated productive infection of macrophages with HHV-7. In addition, HHV-7 has also been detected in normal human tissues by IHC, at higher levels in lung, skin and mammary glands and at lower levels in liver, kidney and tonsils (Kempf *et al.*, 1998). These findings show that the virus remains in human tissues in a persistent form.

1.5.3 Epidemiology and transmission

HHV-7 is ubiquitous and seroprevalence ranges from 60% to over 90% in the healthy adult populations (Black and Pellet, 1999). Like HHV-6, infection with HHV-7 occurs early in life. The peak of seroconversion for HHV-7 occurs after 24 months of age (Tanaka-Taya *et al.*, 1996) and is slightly later than seroconversion for HHV-6 which peak at 13 months (Okuno *et al.*, 1989). Viral loads are also high during this period of acute infection and can reach 10^7 genomes/μg PBMC DNA (Clark *et al.*, 1997). HHV-7 DNA can frequently be detected by PCR in the saliva of both adults and children (Kidd *et al.*, 1996; Tanaka-Taya *et al.*, 1996). Molecular epidemiological evidence for horizontal transmission of HHV-7 also exists (Takahashi *et al.*, 1997a; Thawaranantha *et al.*, 2002). In the study by Takahashi *et al.* (1997a) similar restriction endonuclease patterns of viral DNA purified from saliva isolates of family members living in the same household were found. HHV-7 DNA has also been detected at high frequencies in salivary gland tissue (Di Luca *et al.*, 1995; Sada *et al.*, 1996), and viral protein was detected in the acini and the epithelial cells (Yadav *et al.*, 1997). Thus, salivary glands appear to be the reservoir of persistent HHV-7 replication and saliva is the most likely route of viral transmission. In addition, HHV-7 has been detected by PCR in 3% of the cervixes of women in the later stages of pregnancy (Okuno *et al.*, 1995) suggesting the potential for perinatal transmission. HHV-7, but not HHV-6 has also been detected in 3 of 29 breast milk samples, breast feeding may therefore be another route of HHV-7 transmission (Fujisaki *et al.*, 1998).

1.5.4 Primary infection

Similar to HHV-6, primary infection usually occurs during childhood, with majority of children infected by 3 years of age (Clark *et al.*, 1993). Early reports have suggested that the primary aetiological agent of ES is HHV-6B (Yamanashi *et al.*, 1988). Recent studies have also indicated that HHV-7 may be an additional causative agent of ES (Tanaka *et al.*, 1994; Asano *et al.*, 1995). However, several serological studies have indicated that most children acquire HHV-6 prior to HHV-7 (Tanaka-Taya *et al.*, 1996; Caserta *et al.*, 1998) suggesting that reactivated HHV-6 may be a potential cause of the clinical symptoms. An association between HHV-7 and Pityriasis rosea, a relapsing rash illness, has also been described (Drago *et al.*, 1997) although results have not been confirmed (Kempf *et al.*, 1999). A recent case of delayed primary HHV-7 infection in a 19 year old immunocompetent individual with encephalitis has also been reported (Ward *et al.*, 2002).

1.5.5 HHV-7 latency and persistence

Like the other β-herpesviruses, after primary HHV-7 infection, the virus is able to establish lifelong persistence. Similar to HHV-6, HHV-7 DNA can be detected in the PBMC of healthy immunocompetent individuals using nested PCR suggesting low levels of latent virus in peripheral blood (Kidd *et al.*, 1996). In contrast, HHV-7 viral loads are maintained at high levels in saliva of seropositive individuals (Fujiwara *et al.*, 2000). As mentioned above, salivary glands are a major site of replication following primary infection, and saliva the mode of virus transmission.

1.5.6 HHV-7 infection following transplantation

Yalcin *et al.* (1994) used virus isolation, PCR and serological assays to investigate the prevalence of HHV-7 infection in renal transplant patients compared to that in a control group. The prevalence of HHV-7 DNA was found to be the same in patients and controls (19%). However, the mean Ab titre of HHV-7 in patients was significantly higher than that of the matched healthy controls (1:171 versus 1:49), suggesting that HHV-7 primary infection or reactivation occurring at some time during the post-transplantation period. A direct causal association between HHV-7 and clinical illness in the transplant setting has not yet been established. HHV-7 has been associated with encephalitis in BMT and hepatitis in liver transplant patients (Griffiths *et al.*, 1999a;

Chan *et al.*, 1997), although others have failed to detect an association between HHV-7 and engraftment or the incidence of GVHD (Wang *et al.*, 1996; Chan *et al.*, 1997).

Subsequent prospective studies have reported associations between the development of HCMV disease in transplant recipients and concurrent HHV-7 activity (see table 1.10). Osman *et al.* (1996) monitored PBL from 56 renal transplant recipients by PCR for approximately 3 months after transplantation. Twenty eight patients had HCMV DNAemia, and 8 developed HCMV disease. The risk of developing HCMV disease was increased if patients had HCMV and HHV-7 DNA in PBMCs. Development of HCMV disease was also associated with rising Ab titres to HHV-7, and this suggests that HHV-7 may be a cofactor in disease progression. A recent study by Tong *et al.* (2000) investigated 37 renal transplant recipients for the presence of HHV-6 and HHV-7 DNA and serologic responses to these viruses for 12 weeks after renal transplantation. They found that plasma HCMV load and the occurrence of HCMV disease was related to the serologic response to HHV-6 and HHV-7, although only the presence of HHV-7 DNA in PBL and HCMV donor/recipient serostatus were significantly associated with HCMV disease in logistic regression analysis. Consistent with data showing that high HCMV load increases the possibility of HCMV disease, patients with detectable HHV-7 DNA had significantly higher peak plasma HCMV loads. The possibility of HHV-7 affecting the replication of other β-herpesviruses is supported by reports showing that *in vitro* HHV-7 infection can reactivate latent HHV-6 (Katsafanas *et al.*, 1996; Tanaka-Taya *et al.*, 2000).

The association of HHV-7 and graft rejection has also been reported. A prospective study by Kidd *et al.* (2000) carried out in our centre (and which forms the basis of the patient population studied in chapter 4) on 52 renal transplant recipients showed that the median maximum viral load of HCMV to be greater than that for either HHV-6 or HHV-7. There was a trend for HHV-7 maximum viral loads tend to be greater than those of HHV-6 (P=0.08). Clinicopathological analysis showed that patients with HCMV and HHV-7 coinfection were more likely to have HCMV disease than those with HCMV infection only. HHV-7, but not HHV-6 was significantly associated with increased episodes of graft rejection. HHV-7 is a possible cofactor in the development of HCMV disease in renal transplant recipients and may potentially exacerbate graft rejection. A recent report by Lowance *et al.* (1999) showed that prophylactic treatment

with a high dose VACV for 90 days after renal transplantation reduced the risk of acute graft rejection (50% reduction), and this may partly be mediated by the inhibitory effect of this drug on HHV-6 and HHV-7 replication as well as to its effects on HCMV replication.

Study	Transplant type	Method of detection	No. of pts	No. of bloods	Observed disease
Osman et al (1996)	renal	PCR	56	NG	CMV disease
Kidd et al (2000)	renal	PCR	52	296	CMV disease Graft rejection*
Tong et al (2000)	renal	PCR	37	NG	CMV disease
Mendez et al (2001)	liver	PCR	33	NG	CMV disease
Wang et al (1996)	bone marrow	PCR	37	270	None
Chan et al (1997)	bone marrow	PCR	61	563	Delayed engraftment*
Maeda et al (1999)	bone marrow	PCR	38	209	None
* analysis on subgroup of patient population					

Table 1.10 Prospective studies of HHV-7 post-transplantation (adapted from Clark, 2002)

1.5.7 Antiviral susceptibility of HHV-6 and HHV-7

There have been no controlled trials of antiviral therapy against HHV-6 or HHV-7 infection. However, case reports have shown the potential of existing antiviral drugs (GCV and/or PFA) used for HCMV to suppress HHV-6 replication *in vivo* (Johnston *et al.*, 1999; Zerr *et al.*, 2002). Clinical response of HHV-6 encephalitis or other CNS disease after BMT to therapy with either GCV or PFA or both have been reported (Mookerjee and Vogelsang, 1997; Bethge *et al.*, 1999).

It has been shown that the U69 protein of HHV-6 is the homologue of UL97 phosphotransferase, the CMV gene responsible for the activation of GCV to its monophosphate form (Ansari and Emery, 1999; deBolle *et al.*, 2002). Resistance to GCV has been mapped to HHV-6 U69 gene (Manichanh *et al.*, 2001). HHV-7 encodes a gene homologous to HHV-6 U69, although no functional studies on this gene product have been reported. Less is known of HHV-7 susceptibility to antivirals. Some studies have reported that HHV-7 shows a similar degree of susceptibility to GCV as HHV-6 (Takahashi *et al.*, 1997b), whereas other studies have not shown such an effect (Yoshida *et al.*, 1998).

In vitro, HHV-6, which lacks a thymidine kinase, shows little or no susceptibility to thymidine kinase-dependent drugs such as ACV (Takahashi *et al.*, 1997b). However, in a study by Wang *et al.* (1996) fewer HHV-6 PCR positive blood samples were reported in BMT patients receiving high-dose ACV compared with those without the drug. These results suggest that *in vivo*, high doses of ACV may provide some inhibitory effects against HHV-6 replication. In a prospective study of liver transplant recipients, high HHV-6 or HHV-7 viral loads, concomitant with HCMV infection and disease, were reduced by GCV therapy (Mendez *et al.*, 2001). Therefore the observed clinical benefits of these antiviral drugs could result from inhibition of HCMV, HHV-6 or HHV-7.

1.6 Laboratory diagnosis of HCMV infection

All human β-herpesviruses can cause morbidity in organ transplant recipients, but HCMV causes the major burden of disease. Important risk factors have been identified for the potential development of HCMV (end organ) disease. These include D/R serostatus, viraemia (Pillay *et al.*, 1993) and peak viral load following transplantation (Cope *et al.*, 1997a; 1997b; Gor *et al.*, 1998). HCMV viral load is a central factor in HCMV pathogenesis, such that increased HCMV replication is the single most significant risk factor for disease. Therefore the concept of a threshold value of HCMV load can be introduced, with the absence of disease being characterized by a lower or absence of viral load and disease being associated with higher viral load. In some patients, this viraemia could result in the seeding of multiple organs with HCMV infection. Whilst the measurement of HCMV in the blood is a reliable indication of the degree of viraemic dissemination, it does not allow diagnosis of direct HCMV end-organ involvement (for example pneumonitis, hepatitis, and gastrointestinal disease) which requires detection of virus at the tissue level.

This section describes some of the laboratory methods for diagnosis of HCMV infection. The more conventional cell culture methods for detecting viraemia are currently replaced by more rapid cell culture methods including shell vial or detection of early antigen fluorescent foci (DEAFF). Serological assays are of limited value for the diagnosis of active infection after transplantation and during increased intensity of immunosuppression, as significant Ab rises come (too) late and tests for IgM often remain negative. However, HCMV serology is useful for pre-transplant assessment of the recipient's risk of HCMV infection because of the increased risk associated with donor and recipient seropositivity. Molecular techniques such as QC-PCR, and the semiquantitative HCMV pp65 antigenemia assay (Kim *et al.*, 2003) have been used to diagnose HCMV infection and assess the viral load in transplant patients. Recently, new applications based on real-time quantitative PCR technology such as the Lightcycler, or the TaqMan technology have been developed (Mengelle *et al.*, 2003; Li *et al.*, 2003). Blood either whole blood, plasma, or PBMC are used to monitor for infection. However, to study more closely the pathogenesis of HCMV infection and disease, at the organ level, *in situ* techniques must be utilized. Traditionally, the diagnosis of HCMV direct end organ disease has relied on classical histopathological techniques to visualize "owl's eye inclusions" (Smith, 1959; Macasaet *et al.*, 1975) in

the biopsies of the affected organ. However, this method is insensitive (Mattes *et al.*, 2000). Currently, more sensitive and specific methods including IHC and ISH have been developed.

1.6.1 Virus isolation

Isolation of HCMV from tissue or body secretions remains the 'gold standard' against which other tests are compared. For conventional cell culture, clinical specimens are inoculated onto semi-continuous human fibroblast cells obtained from the foreskin or embryonic lung and tube cultures of these cells are observed regularly for the development of HCMV CPE characterized by foci of flat, swollen cells. Development of CPE can take up to 28 days, depending on the titre of the virus in the sample. The long time required for diagnostic confirmation of this method therefore limits its clinical usefulness.

1.6.2 DEAFF and shell-vial assays

More rapid cell culture methods relying on culture confirmation with monoclonal antibodies termed shell vial (Gleaves *et al.*, 1984) or DEAFF (Griffiths *et al.*, 1984) allows detection of virus within 24-48 hrs but is no more sensitive than traditional virus isolation (Boppana *et al.*, 1992b).

1.6.3 Antigenemia assay

The antigenemia assay measures a late structural protein. HCMV pp65 is a 65 kDa lower matrix protein and can be detected in PBL (Revello *et al.*, 1992). The quantitative nature of this assay provides a useful tool for predicting the likelihood of HCMV disease- the presence of a small number of Ag-positive cells following SOT generally indicates asymptomatic infection, whereas a large number implies an increased likelihood of HCMV disease (Niubo *et al.*, 1996; The *et al.*, 1993).

1.6.4 The polymerase chain reaction (PCR)

Qualitative PCR is used for routine monitoring of HCMV in clinical samples. However, qualitative PCR often cannot distinguish between latent and active infection unless a limited input quantity of DNA is tested. In our centre 30 ng of DNA extracted from whole blood is used for our PCR assay, an amount below the threshold where virus is detected in blood of persons with normal immunity. Alternatively, reverse-

transcription (RT)-PCR can be used. Quantitative PCR has been employed to perform longitudinal studies in different patient groups at risk of HCMV disease (Bowen *et al.*, 1998; Cope *et al.*, 1997a; 1997b; Fox *et al.*, 1995; Hassan-Walker *et al.*, 1999).

1.6.5 *In situ* techniques

1.6.5.1 Histopathology

The diagnosis of HCMV organ disease has traditionally been based upon histologic recognition of cytomegalic inclusion bodies with the characteristic intranuclear 'owl's eye' appearance in hematoxylin and eosin (H and E)-stained tissue specimens. In most cases, the detection of viral inclusion bodies in liver tissue correlates with symptomatic HCMV disease. However, histopathology is known to be insensitive although it provides a specific diagnosis. In a study correlating HCMV load in tissues obtained at postmortem examination of AIDS patients with the histological evidence of HCMV inclusions, a viral load of >5 000 000 genomes/μg DNA was required before HCMV inclusions were observed (Mattes *et al.*, 2000).

1.6.5.2 Immunohistochemistry (IHC)

The detection of HCMV Ags with MAbs has increased the sensitivity for the histologic diagnosis of CMV disease in transplant patients compared to standard H and E staining (Barkholt *et al.*, 1994; Paya *et al.*, 1990).

1.6.5.2.1 Technique of IHC

The principle steps of IHC include (1) preparation of the biological material, (2) Antigen unmasking, (3) Ab staining, and (4) Ab detection (see figure 1.5). Preparation of cells and tissue sections for IHC (and ISH) involves routine fixation to ensure the preservation of tissue architecture and cell morphology. The predominant method of tissue preservation for microscopic analysis is by fixation with a formaldehyde-based fixative, followed by tissue processing for paraffin embedding. The development of Ag unmasking (Shi *et al.*, 1991) has helped improve the sensitivity of immunostaining. This technique uses a combination of high temperature and buffers of different pH (the most commonly used is 0.1M Citrate buffer, pH 6.0), and recently the introduction of non-heating methods, including enzymatic digestion (Shi, 2001) have also been applied. There are two methods of Ab staining. The direct method involves a labeled Ab reacting with the Ag in the tissue followed by the subsequent addition of substrate-

chromagen. This procedure is short and quick but relatively insensitive. This led to more sensitive indirect methods involving an unlabeled primary Ab which reacts with tissue Ag and a labelled secondary. Ab staining methods frequently utilize enzyme coloured reactions to convert chromagens into coloured end products. Peroxidase is the commonly used enzyme label and the substrates for peroxidase include 3-amino-9 ethyl carbazole (AEC), which gives a red precipitate and 3,3-diaminobenzidine (DAB) which results in an intense brown precipitate.

<table>
<tr><td>Immunohistochemistry</td><td>In situ hybridisation</td></tr>
<tr><td colspan="2">Tissue preparation</td></tr>
<tr><td>Antigen unmasking
a) high temp and buffers
b) Enzymatic digestion</td><td>Unmasking to target DNA/RNA
a) dilute acid (e.g HCl)
b) detergent
c) proteolytic enzymes</td></tr>
<tr><td rowspan="2">Antibody staining either:-
1) Direct method
or
2) Indirect method</td><td>Hybridisation with labelled probe</td></tr>
<tr><td>Post-hybridisation washes</td></tr>
<tr><td colspan="2">Detection with chromogenic substrate</td></tr>
</table>

Figure 1.5 Diagram of immunohistochemistry and *in situ* hybridisation technique

Methods for amplification of the detection system have also been adopted to increase the sensitivity of IHC. The most widely used technique is the avidin-biotin complex (ABC) method developed by Hsu *et al.* (1981). It is based on the high affinity and essentially irreversible interaction between avidin and biotin. The technique requires pre-incubation of the biotinylated enzyme with the avidin, so that large complexes are formed for incubation with the biotinylated secondary Ab (see figure 1.6a). More recently, the application of chain-polymer technology such as DAKO Envision Systems has been employed. In the Envision System the secondary Ab is conjugated to the enzyme labeled polymer (see figure 1.6b) which contains an average of 10 molecules of secondary Ab and 70 molecules of enzyme, thereby increasing the sensitivity of detection.

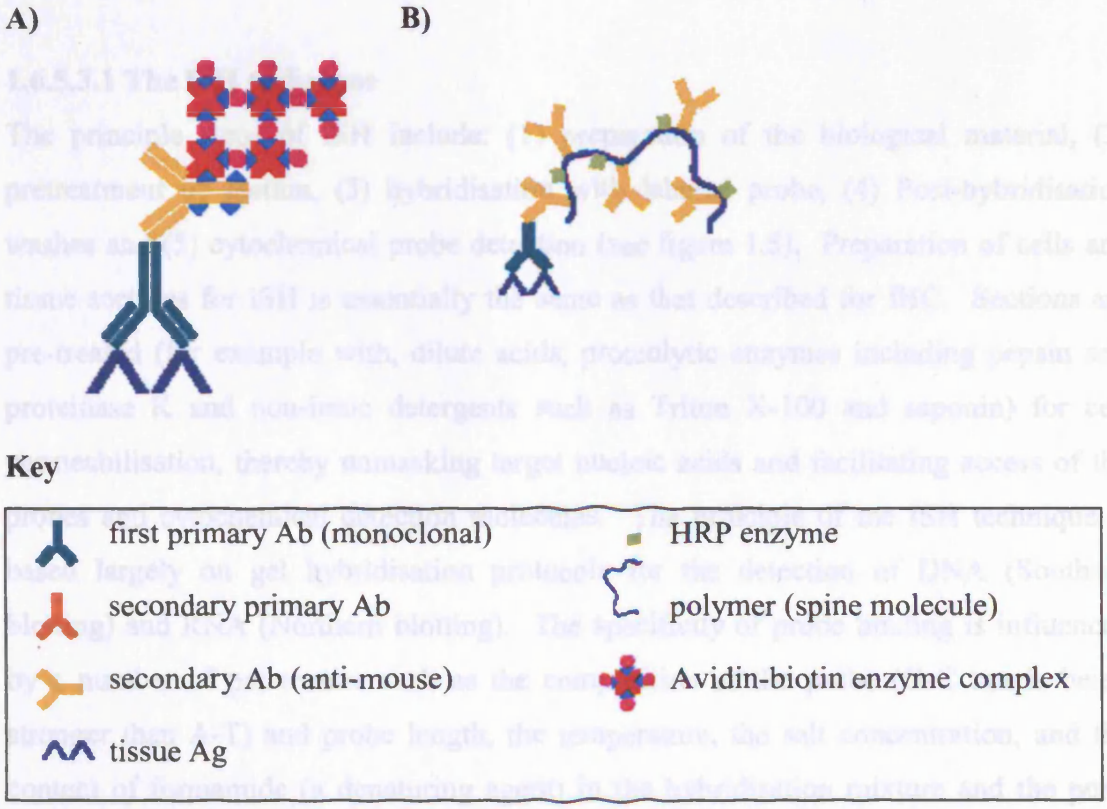


Figure 1.6 Amplification of detection systems A) The ABC method: the avidin-biotin complex reacts with the biotinylated secondary Ab; B) The Envision System: the spine molecule which contains an average of 10 molecules of secondary Ab and 70 molecules of enzyme (adapted from www.dako.com).

1.6.5.3 *In situ* hybridisation

In situ hybridisation (ISH) is a technique for localization of specific nucleic acid sequences in microscopic preparations of tissues, cells or chromosomes. It was originally developed in 1969 by Pardue and Gall and (independently) by John *et al.* and is based on the complementary binding of nucleic acid probe to a specific target sequence of DNA or RNA. Hybridisation has conventionally been performed with radioisotopically labeled probes followed by autoradiographic probe detection. This has largely been replaced by non-radioactive probe labeling and detection systems (Rudkin and Stoller, 1977). ISH has been applied for the diagnosis of HCMV pneumonitis (Gleaves *et al.*, 1989; Solans *et al.*, 1995), hepatitis (Masih *et al.*, 1988; Colina *et al.*, 1995; Paya *et al.*, 1990) and gastroenteritis (Muir *et al.*, 1998). One of the limitations of ISH has been the inability of ISH to discriminate between active infection and latency (Ehrnst, 1996). The introduction of probes specific for HCMV IE mRNA, however has allowed for the identification of early HCMV infection (Link *et al.*, 1993).

1.6.5.3.1 The ISH technique

The principle steps of ISH include: (1) preparation of the biological material, (2) pretreatment of section, (3) hybridisation with labeled probe, (4) Post-hybridisation washes and (5) cytochemical probe detection (see figure 1.5). Preparation of cells and tissue sections for ISH is essentially the same as that described for IHC. Sections are pre-treated (for example with, dilute acids, proteolytic enzymes including pepsin and proteinase K and non-ionic detergents such as Triton X-100 and saponin) for cell permeabilisation, thereby unmasking target nucleic acids and facilitating access of the probes and cytochemical detection molecules. The principle of the ISH technique is based largely on gel hybridisation protocols for the detection of DNA (Southern blotting) and RNA (Northern blotting). The specificity of probe binding is influenced by a number of parameters such as the composition of the probe (G-C bonds being stronger than A-T) and probe length, the temperature, the salt concentration, and the content of formamide (a denaturing agent) in the hybridisation mixture and the post-hybridisation washes. Four types of probes can be used for ISH: double stranded complementary DNA (cDNA), single stranded cDNA, RNA probe (riboprobe) and oligonucleotides (reviewed in Dirks, 1996). Probes can be labeled directly (usually with fluorescein or fluorochromes) or indirectly where a reporter molecule (such as biotin or digoxigenin (DIG), introduced chemically or enzymatically, can be detected by IHC

techniques. Detection of probe is usually carried out with enzyme conjugated Abs raised against the label (DIG or biotin) (reviewed in Speel, 1999).

Aims of thesis

1) To develop *in situ* techniques including *in situ* hybridisation and immunohistochemistry for the detection of the human β-herpesviruses.

2) To apply these optimized assays to examine the presence of these viruses (viral DNA or specific antigens) in organ biopsies from previous prospective studies of liver and renal transplant patients, and to investigate the role of these viruses in graft dysfunction, including allograft rejection.

3) To examine for evidence of both vascular (by C4d staining) and cellular graft rejection in the renal transplant patients and to correlate the presence of rejection with HCMV status in the biopsy.

4) To construct a riboprobe specific to a region of the HCMV immediate early-1 gene for ISH to investigate HCMV gene expression at the mRNA level in the renal biopsies following transplantation.

5) To study HHV-6 integration into host cell chromosomes by the development of fluorescence *in situ* hybridisation.

Chapter 2 - Materials and Methods

2.1 Cell culture and virus propagation

Section 2.1 describes cell culture methods for the preparation of cell controls for subsequent *in situ* methods.

2.1.1 Cell culture

MRC-5 and human embryonic lung (HEL) fibroblasts were grown in minimum essential medium (MEM) (Invitrogen) supplemented with 10% foetal calf serum (FCS) (Labtech International) and 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin solution (Sigma) in a 75 cm^2 tissue culture flask at 37^0C. Once the cells reached confluency, the media was removed, and the cell monolayer was washed with 10 ml phosphate-buffered saline (PBS). The PBS was removed and 3 ml of trypsin (0.05%)/EDTA (0.01%) (Invitrogen) were added to the flask. The flask was placed at 37^0C for approximately 3 min until the cells had detached from the surface, and could be loosened with gentle tapping. Ten millilitres of growth medium were then added to the flask and mixed. The cells were then split into two 75 cm^2 flasks. Approximately 5 ml cell culture was added to each flask and growth media was added to a final volume of 30 ml. The cells were incubated at 37^0C for up to a week. Cells were passaged twice weekly.

2.1.2 Virus propagation

The laboratory strain of HCMV, AD169 was cultured in MRC-5 or HEL cells. The cells were grown to 80-90% confluency in 30 ml growth media in a 75 cm^2 flask. The medium was removed, and the cell monolayer was washed with 10 ml PBS. The PBS was removed, and 5 ml of stock AD169 were inoculated onto the cells and allowed to adhere to the cells for 1 hr in a 37^0C incubator. The viral inoculum was removed, the cell monolayer was washed with maintenance media (MEM media supplemented with 5% FBS and Pen/Strep), and replaced with 10 ml fresh maintenance media. The cells were incubated in a 37^0C incubator for approximately 1 week or until CPE was observed. The supernatant containing the virus was then removed and centrifuged at 400 *g* for 5 min to remove the cell debris. The supernatant was aliquoted into 2 ml tubes and stored frozen at -70^0C. Another 10 ml maintenance media was added to the flask, and the cells were incubated at 37^0C for a further 24 hrs. Viral stock supernatant

was collected from the cells as described above, until there were no more adherent cells visible.

HHV-6B (strain Z29) infected and non-infected Molt-3 cell cultures and HHV-7 (DC strain) infected and non-infected Sup T1 cell cultures were obtained from Dr Duncan Clark and Edward Tsao, Department of Virology, Royal Free and University College Medical School.

2.1.3 Cell preparations

Cells from infected cultures; HCMV (strain AD169) infected HEL/MRC-5, HHV-6B (strain Z29) infected Molt-3, and HHV-7 (DC strain) infected Sup T1 cells were grown, and harvested for use as positive controls for optimisation of *in situ* techniques. Generally, cell cultures were grown until over 50% of cells were infected with virus based on visualisation of CPE. HCMV (strain AD169) infected MRC-5 cells were also grown for 3-4 hrs to limit expression of viral genes to IE only. Non-infected MRC-5, HEL, Molt-3 and Sup T1 cell cultures were used as negative controls.

2.1.3.1 Fixation of cells to glass slides

Five to 10 ml cells from virus infected and non-infected cultures were harvested by centrifuging at 400 *g* for 5 min. The supernatant was removed, and the cell pellet was washed in 10 ml PBS three times to remove any serum proteins, before resuspending in 200 µl PBS. The number of cells was counted using a haemacytometer and approximately $1x10^5$ cells/50µl PBS were spotted onto aminopropyl tri-ethoxysilane (APES) coated glass slides (Applied Biosystems). The cell suspension was left to air dry for approximately 1 hr before fixing in cold 4% paraformaldehyde in PBS for 20 min. Fixation helps to reduce nucleic acid loss and preserve cellular morphology. The slides were then washed twice in PBS for 5 mins each, dehydrated in increasing concentrations of ethanol (25%, 50%, 75% and 100%) and stored at -20^0C until ready for use.

2.1.3.2 Preparation of cell suspensions for paraffin-embedding

Ten to 20 ml of virus infected and non-infected cultures were harvested by centrifuging at 400 *g* for 10 min. The supernatant was removed, and the pellet was washed in 10 ml PBS twice. The cell pellet was then transferred to a 1.5 ml microfuge tube, and an approximately equal volume of melted 3% (w/v) low melting temperature agarose (Sigma) in 1X TBE buffer (90 mM Tris-borate, 2mM EDTA), cooled to 50^0C, was added to the pellet and mixed thoroughly by pipetting up and down. The agarose plug was left to harden for approximately 30 min before transferring and wrapping in Biowrap, and then fixing in 4% neutral buffered formalin (CellStor pots; Cellpath plc) overnight, followed by paraffin-embedding. Using a microtome blade (Leica RM 2035), 4 µm sections were cut, floated on distilled H_20 in a 37^0C waterbath and mounted onto APES-coated glass slides. The sections were then baked in a hybridisation oven at 55^0C for 48 hr to aid the adhesion of cells onto the slides.

2.2 Development of immunohistochemistry

Immunohistochemistry (IHC) is a technique that uses Abs to detect, visualize and localize Ags at the cellular level. Section 2.2 describes IHC using human β-herpesvirus MAbs for the detection of viral specific proteins. Two amplification detection methods were used for the development of IHC; the avidin-biotin peroxidase complex method described in section 2.2.1, and the ChemMate Envision detection system (DakoCytomation Ltd) described in section 2.2.2.

2.2.1 The avidin-biotin peroxidase complex method

The avidin-biotin peroxidase complex method was originally introduced by Hsu *et al.* (1981). This patented procedure uses biotinylated Ab and a preformed Avidin Biotinylated enzyme Complex and has been termed the "ABC" technique. Avidin is a tetramer consisting of four identical subunits of molecular weight 15, 000 Da. Each subunit contains a high affinity binding site for biotin with a dissociation constant of approximately 10^{-15}M. This affinity for biotin is over one million times higher than that of Ab for most Ags, and so unlike Ab-Ag interactions, the binding of avidin to biotin is essentially irreversible. The Vectastain Elite ABC kit (Vector labs) contains Avidin DH and biotinylated enzyme, and was used to form ideal complexes for immunohistochemical staining of the human β-herpesvirus proteins.

2.2.1.1 IHC on paraformaldehyde-fixed cell suspensions

IHC was performed with MAbs directed against HCMV, HHV-6 and HHV-7 on respective paraformaldehyde-fixed infected cell suspensions. MAb to HCMV late protein of 47-55 kDa (MAB8126; Chemicon International), MAb to HHV-6B virion protein of 101K (p101) (MAB8535; Chemicon International), and MAb 5E1 to HHV-7-specific phosphoprotein 85 (pp85) (kindly provided by Dr Gabriella Campadelli-Fiume, University of Bologna, Italy (Stefan *et al.*, 1997) were tested at a range of dilutions (1:100-1:500) on HCMV infected HEL cells, HHV-6B infected Molt-3 cells and HHV-7 infected Sup T1 cells respectively. Non-infected HEL, Molt-3, and Sup T1 cells were used as negative controls.

Using a pap pen (Zymed labs), a circle was drawn around the cell suspension to help retain reagents on the glass slide. The slides were immersed in 100% ethanol and then 100% methanol. Endogenous peroxidase (which will produce a reaction product from the substrate alone) was blocked by incubating the slides with 1.5% Hydrogen Peroxide (30% w/w solution) (Sigma) in 100% methanol (BDH) for 30 min. The slides were then rehydrated through decreasing concentrations of ethanol washes (100%, 75%, 50% and 25%) to distilled H_20 for 2 min each. Primary Ab in first layer diluent (0.3% Triton X-100, 2% normal horse serum, 0.01% sodium azide in PBS) was subsequently added to the sections, and the slides were placed in a humidified chamber for incubation overnight at 4^0C. After overnight incubation in primary Ab, the slides were washed in PBS three times for 5 min each. Secondary biotinylated anti-mouse IgG (Vector labs) at 1:100 dilution in second layer diluent (0.3% triton X-100 in PBS) was added to the slides, and incubated in a humidified chamber for 50 min at room temp. The slides were washed in PBS three times for 5 min each, and incubated in a third layer complex (Vectastain Elite ABC kit, Vector labs) for 1 hr at room temp. The third layer complex was prepared according to the maufacturer's instructions. Two drops of reagent A, and two drops of reagent B, were added to 5 ml second layer diluent with mixing. This was prepared 30 min before use. After PBS washes three times for 5 min each, the peroxidase substrate was detected using DAB substrate kit (Vector Labs). Two drops of Buffer Stock Solution, 4 drops of DAB Stock Solution and 2 drops of Hydrogen Peroxide were added to 5 ml of distilled H_20, mixing well each time. The slides were incubated in DAB substrate solution for 10 min. DAB produces a reddish brown precipitate in the sections. The reaction was stopped by washing the slides in distilled H_20 for 5 min, followed by dehydration in increasing ethanol concentrations (25%, 50%, 75% and 100%). The slides were air dried, and mounted with UV Loctite 358 adhesive (Loctite UK Ltd). The adhesive was applied to the centre of the slide, and a glass cover slip was placed on top. The slides were cured by placing onto the UV transilluminator for 1 min. The slides were subsequently monitored by viewing under a light microscope (Olympus BX60).

2.2.1.2 IHC on formalin-fixed paraffin-embedded cell suspensions

The same three MAbs directed against HCMV, HHV-6 and HHV-7 as described in section 2.2.1.1 were evaluated by performing IHC on formalin-fixed paraffin-embedded cell controls; HCMV infected HEL, HHV-6B infected Molt-3 and HHV-7 infected Sup T1 cells respectively. Negative controls of non-infected HEL, Molt-3 and SupT1 cells were included in each staining run. An additional three MAbs directed against HHV-6 proteins were also tested. These include; MAb to HHV-6 early protein p41/38, MAb to HHV-6 gp116/64/54 (referred to as HHV-6 gB (Ellinger *et al.*, 1993), and MAb to HHV-6B p98 (also known as MAb OHV3) all obtained from Advanced Biotechnologies International. MAb OHV3 reacts with a HHV-6B-specific neutralizing epitope on gH (Takeda *et al.*, 1997) and so will be referred to as MAb to HHV-6B gH. Preliminary experiments were performed using a range of dilutions (1:50-1:100) of the MAbs to determine the dilution required to detect human β-herpesvirus Ags in formalin-fixed paraffin-embedded virus infected cells, without a background reaction. IHC was carried out as described in section 2.2.1.1 with an initial deparaffinization step in xylene twice for 15 min at room temp before immersing in 100% ethanol.

2.2.1.3 Incorporation of an antigen retrieval method into IHC

Staining was refined by incorporating an Ag retrieval method. Ag retrieval helps break the protein cross-links formed by formalin fixation and thereby allowing the availability of the Ag for interaction with a specific Ab to be maximized. Two methods were evaluated; enzymatic digestion and microwave irradiation.

The MAbs described in section 2.2.1.1 and two additional MAbs directed to the HCMV delayed early DNA-binding protein p52 (CCH2 MAb; Dako) (Plachter *et al.*, 1992), and to the HCMV IE non-structural protein of 68-72 kDa (MAB8131; Chemicon) were all independently evaluated at a range of dilutions (1:100-1:500) on formalin-fixed paraffin-embedded cells infected with the respective virus. Microwave irradiation, or an enzymatic digestion step was also incorporated into IHC. For comparison, IHC with no Ag retrieval method was performed in parallel. Non-infected cells were used as negative controls.

The sections were deparaffinized twice in xylene for 15 min, endogenous peroxidase was blocked as described in section 2.2.1.1 followed by rehydration of the sections to distilled H_20. For enzymatic digestion, slides were incubated at a range of Proteinase K (Sigma) concentrations ranging from 2.5 µg/ml-10 µg/ml in Proteinase K buffer (50 mM Tris-HCl, 5 mM EDTA pH 7.6) for 30 min at 37^0C, followed by washes in PBS three times for 5 min. For microwave irradiation, sections were boiled in an 800 watt microwave (Hinari) for 5 min in preheated citrate buffer (0.01M citric acid, pH 6.0), and cooled for a further 25 min at room temp. The slides were then washed in PBS three times for 5 min each. After the Ag retrieval step, immunohistochemical detection was continued as described in section 2.2.1.1.

2.2.1.4 Incorporation of an avidin biotin block into IHC

Once the optimal concentration of MAb, and the Ag retrieval method had been determined, an avidin biotin blocking kit (Vector Labs) for reducing background levels was tested. This blocking kit consists of an Avidin D solution and a Biotin solution. Background may result from some tissue sections binding avidin, biotinylated horseradish peroxidase or other Biotin/Avidin System components without prior addition of biotinylated Ab. This binding may be due to endogenous biotin or biotin-binding proteins, lectins, or non-specific binding substrates present in the section. MAbs directed against HCMV proteins characteristic for each stage of HCMV replication; MAB8131 to HCMV IE protein, CCH2 MAb to HCMV p52, and MAB 8126 to HCMV late protein were used for development of IHC with the incorporation of an avidin biotin block. In addition, MAb to HHV-6 gB, MAB8535 to HHV-6B p101 and MAb 5E1 to HHV-7 pp85 were also tested. For comparison, the MAbs were tested at the same dilutions without the blocking step. Non-infected cells were used as negative controls. The sections were deparaffinized, endogenous peroxidase was blocked, and an Ag retrieval method was incorporated as previously described. The slides were then incubated with 10% normal horse serum in PBS with Avidin D solution (4 drops Avidin D solution per 1 ml 10% normal horse serum in PBS) for 10 min. After a brief wash in PBS, the primary Ab diluted in first layer diluent with the biotin solution was added (4 drops of the biotin solution per 1 ml of primary Ab solution). IHC was continued as described in section 2.2.1.1.

2.2.2 The DAKO ChemMate Envision Detection system

The application of chain-polymer technology (Dako Envision Systems) was also employed to increase the sensitivity of the detection systems. The EnVision detection reagent in the DAKO ChemMate EnVision detection kit, peroxidase/DAB (Dako) consists of a dextran backbone to which a large number of enzyme molecules and secondary Ab molecules have been coupled. A unique chemistry is used for the coupling reaction, which permits the binding of up to 100 enzyme molecules and up to 20 Ab molecules per backbone. Endogenous biotin also has no affect on staining results.

The MAbs directed to HCMV described in section 2.2.1.4 were used for development of IHC using the DAKO ChemMate Envision Detection system. IHC was performed on formalin-fixed paraffin-embedded MRC-5 cells infected with HCMV (AD169). Non-infected MRC-5 cells were used as negative controls.

The sections were deparaffinized in xylene twice for 15 min and then rehydrated in decreasing concentrations of ethanol to distilled H_20. Ag retrieval was performed as described in section 2.2.1.3. In addition, both enzymatic digestion and microwave irradiation were incorporated. First, the sections were boiled in preheated 0.01M citrate buffer (pH 6.0) for 5 min in a microwave, cooled for 20 min at room temp followed by incubation with 7.5 μg/ml Proteinase K for 15 min at 37^0C. After washes in PBS three times for 5 min, the slides were incubated with HCMV MAbs (1:50 and 1:100 dilution) for 30 min at room temp. After a 5 min wash in PBS, endogenous peroxidase was blocked by incubation with peroxidase blocking solution (Dako) for 10 min, followed by PBS wash for 5 min. The tissue sections were then incubated with DAKO chemMate Envision/horseradish peroxidase (HRP), rabbit/mouse for 25 min at room temp. After a 5 min wash in PBS, the DAB-containing Substrate Working Solution (50 parts of Substrate Buffer with 1 part of DAB chromogen) was added to the sections for 5 min. The slides were then washed in H_20 for 5 min, air dried, and mounted in UV Loctite 358 adhesive.

2.2.3 Testing the specificity of the human β-herpesvirus MAbs

The specificity and crossreactivity of the immunohistochemical reactions with the MAbs described in section 2.2.1.4 were tested on formalin-fixed paraffin-embedded cells infected with respective virus and to each of the other human β-herpesvirus infected and non-infected cell controls. Methods were as described in section 2.2.1.4 and 2.2.2).

2.2.4 Subtyping of HHV-7 MAb 5E1

At the time these experiments were carried out the Ab isotype of MAb 5E1 to HHV-7 pp85 had not been determined. To characterize the Ab isotype of MAb 5E1, HHV-7 infected Sup T1 cells were incubated with MAb 5E1, followed by the addition of an enzyme conjugated antimouse Ab corresponding to the common mouse isotypes IgG_1, IgG_{2a} and IgG_{2b}, and subsequent immunohistochemical detection using the ABC method.

Formalin-fixed paraffin-embedded HHV-7 infected Sup T1 cells were deparaffinized in xylene, endogenous peroxidase was blocked, and the sections were rehydrated as previously described. The slides were microwaved for 5 min in preheated citrate buffer and cooled for a further 25 min followed by washes in PBS three times for 5 min. MAb 5E1 was added at 1:100 dilution in first layer diluent and incubated in a humidified chamber at 4^0C overnight. Slides were then washed in PBS three times for 5 min each, and secondary Ab (antimouse IgG_1 (AP273), IgG_{2a} (AP274) and IgG_{2b} (AP275) conjugated with peroxidase (The Binding Site Limited) were added at 1:20 and 1:50 dilution in second layer diluent for 1 hr at room temp. Slides were then washed in PBS three times for 5 min each and detection was carried out with DAB substrate as previously described. The reaction was stopped by washing the slides in H_20 for 5 min. The sections were then dehydrated, air dried and mounted with UV Loctite 358 adhesive. HHV-6 infected Molt-3 cells incubated with mouse IgG_{2b} to HHV-6 gB was used as a positive control. HHV-7 infected Sup T1 cells incubated without MAb 5E1, and non-infected Sup T1 cells were included as negative controls.

2.3 Construction of digoxigenin labelled riboprobes for ISH

This section describes the synthesis of digoxigenin (DIG) labelled riboprobes (RNA probes) specific for each of the β-herpesviruses for the detection of viral DNA by ISH (see figure 2.1). In section 2.3.1, the generation of HCMV, HHV-6 and HHV-7 DNA sequences by PCR is described. The amplicons were then purified and cloned into the polylinker site of the transcription vector pGEM-T Easy which contains a promoter for SP6 and T7 RNA polymerases (see sections 2.3.3-2.3.7). Section 2.3.8 describes the linearization of the plasmids prior to the transcription reaction so that only discrete "run-off transcripts, virtually free of vector sequences are obtained. *In vitro* transcription incorporating a DIG RNA labelling mix was then carried out with RNA polymerases, Sp6 and T7 to generate DIG labelled RNA transcripts.

2.3.1 Generation of HCMV, HHV-6 and HHV-7 DNA by PCR

The pUC13 plasmid containing the 149 bp insert of HCMV gB gene control sequence was used as a template for cloning into the transcription vector pGEM-T Easy (Promega). The control sequence is identical to the authentic HCMV gB target sequence with the exception of a 2 bp change at bases 77 and 78 of the 149 bp sequence converting the original GG to a TT. This results in the introduction of a unique *Hpa*I restriction site into the 149 bp target sequence (Fox *et al.*, 1992). The pUC18 plasmids containing the 223 bp insert of HHV-6 unique (U)-67 gene control sequence (Clark *et al.*, 1996), and the 183 bp insert of the HHV-7 U42 gene control sequence (Kidd *et al.*, 1996), with a *Sma*I restriction site in the middle of the sequences, were constructed in the same way as that of Fox *et al.* (1992). These plasmids were used as templates for cloning into the pGEM-T Easy vector.

The control plasmids at a concentration of 10^8 copies were used as templates for PCR amplification. The primers HCMV gB1 and HCMV gB2, HHV-6C and HHV-6D, and HHV-7 MK3 and HHV-7 MK4 were used to generate a 149 bp, a 173 bp and a 143 bp fragment of HCMV gB, HHV-6 U67, and HHV-7 U42 respectively (see table 2.1). In addition, DNA extracted from HCMV (AD169) infected HEL cells were used as template for PCR amplification of a 298 bp sequence of HCMV US28 using primers US28F and US28R (Beisser *et al.*, 2001), and cloned into the pGEM-T Easy vector (see table 2.1 for US28 primers).

Gene	Primer sequence
HCMV gB1	5'-GAG GAC AAC GAA ATC CTG TTG GGC A -3'
HCMV gB2	5'-GTC GAC GGT GGA GAT ACT GCT GAG G -3'
HCMV US28F	5'-CGT CGG ATT CAA TGC TCC GGC GAT GTT TAC-3'
HCMV US28R	5'-GAA TGG CGA TGA TCA CGG CAA AGA TCC ACC-3'
HHV-6 C	5'-TCC ATT ATT TTG GCC GCA TTC GT-3'
HHV-6 D	5'-TGT TAG GATATA CCGATG TGC GT-3'
HHV-7 MK3	5'-TGC TTT TTG GTT TGT AAA TTC -3'
HHV-7 MK4	5'-GAA TTT ATG GAG TTT GGT CTG -3'

Table 2.1 Primer sequences for HCMV, HHV-6 and HHV-7 PCR

Each reaction was performed in triplicate, in 1X PCR buffer containing 2 mM $MgCl_2$, 200 µM each dNTP (dATP, dGTP, dCTP, dTTP) (Promega), 2.5 U of HotStar Taq DNA Polymerase (Qiagen) and 100 ng of each primer in a volume of 98 µl made up with distilled H_20. Two microlitres of plasmid template were added, to a final reaction volume of 100 µl. Negative H_20 controls were included to determine the presence of any contaminating DNA. The PCR cycling conditions for HCMV gB are shown below. The PCR cycling conditions for HHV-6 U67 and HHV-7 U42 were the same as that of HCMV gB, except the annealing temperature was 50^0C. Cycling was performed on a Hybaid DNA thermal cycler.

Hot start	95^0C for 15 min	}	1 cycle
Denaturation	94^0C for 30 sec		
Annealing	60^0C for 30 sec		39 cycles
Extension	72^0C for 30 sec		
Final extension	72^0C for 10 min	}	1 cycle.

The resulting PCR amplicons were detected by electrophoresis on a 3% (w/v) agarose gel.

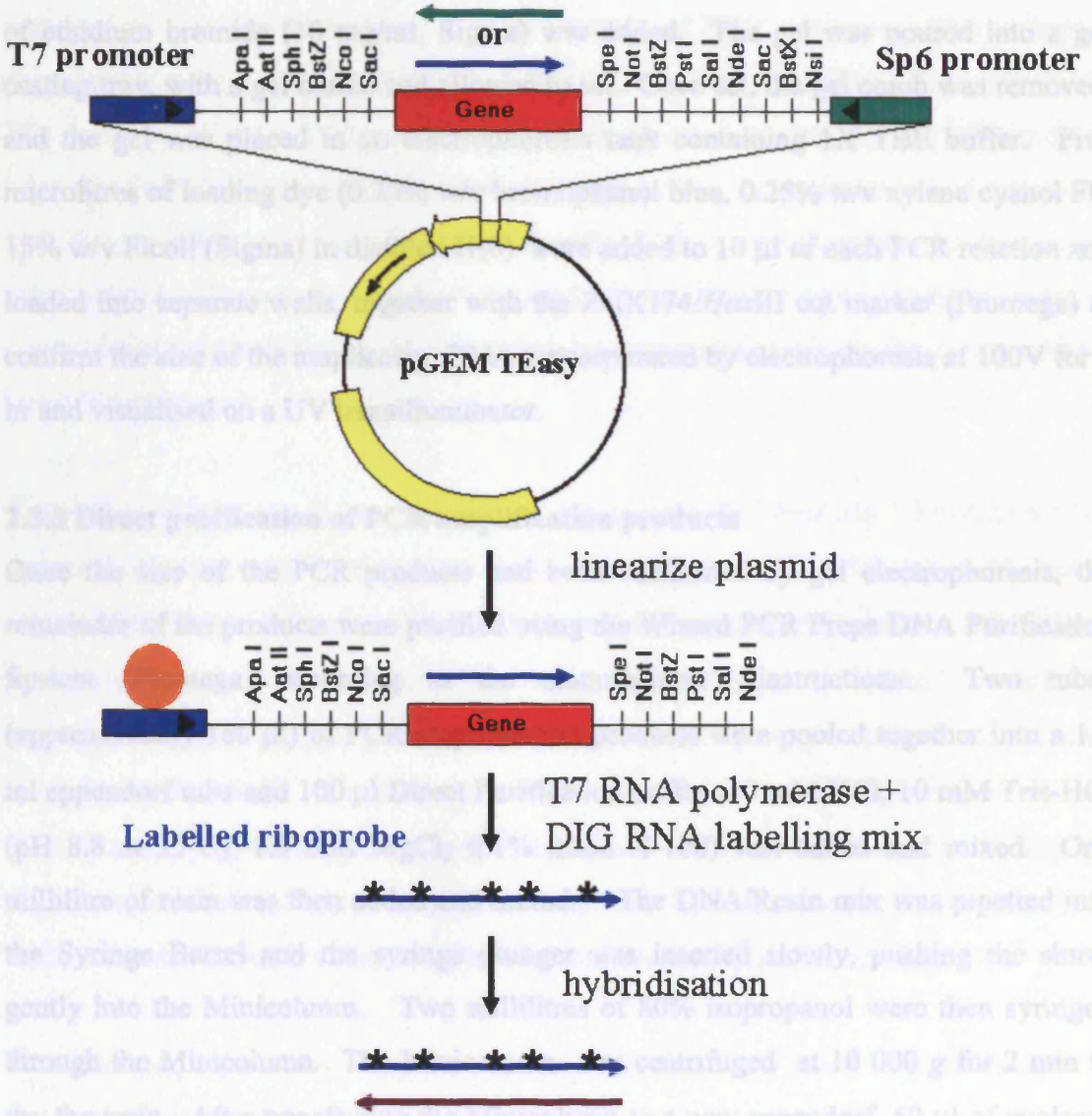


Figure 2.1 Diagram showing riboprobe *in vitro* transcription. Recombinant plasmid DNAs were linearized prior to *in vitro* transcription. *In vitro* transcription incorporating a DIG RNA labelling mix was carried out with RNA polymerases to generate DIG labelled riboprobes for ISH.

2.3.2 Gel preparation and electrophoresis

Three grams of multipurpose agarose (Bioline) in 100 ml of 1X TBE buffer was melted in a microwave oven. The gel was allowed to cool to approximately 50^0C, before 3 µl of ethidium bromide (10 mg/ml, Sigma) was added. The gel was poured into a gel casting tray, with a gel comb, and allowed to set. Once set, the gel comb was removed, and the gel was placed in an electrophoresis tank containing 1X TBE buffer. Five microlitres of loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 15% w/v Ficoll (Sigma) in distilled H_20) were added to 10 µl of each PCR reaction and loaded into separate wells, together with the PhiX174/*Hae*III cut marker (Promega) to confirm the size of the amplicons. DNA was separated by electrophoresis at 100V for 1 hr and visualised on a UV transilluminator.

2.3.3 Direct purification of PCR amplification products

Once the size of the PCR products had been confirmed by gel electrophoresis, the remainder of the products were purified using the Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions. Two tubes (approximately 180 µl) of PCR amplification products were pooled together into a 1.5 ml eppendorf tube and 100 µl Direct Purification buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8 at 25^0C), 1.5 mM $MgCl_2$ 0.1% triton X-100) was added and mixed. One millilitre of resin was then added and mixed. The DNA/Resin mix was pipetted into the Syringe Barrel and the syringe plunger was inserted slowly, pushing the slurry gently into the Minicolumn. Two millilitres of 80% isopropanol were then syringed through the Minicolumn. The Minicolumn was centrifuged at 10 000 *g* for 2 min to dry the resin. After transferring the Minicolumn to a new eppendorf, 50 µl of nuclease free H_20 was applied, and centrifuged at 10 000 *g* for 30 sec to elute the DNA fragment. The concentration of purified DNA was measured spectrophotometry using the Gene Quant spectrophotometer (Pharmacia, Biotech) and stored at -20^0C for future use.

2.3.4 Ligation of HCMV, HHV-6 and HHV-7 DNA into the pGEM-T Easy vector

The purified PCR products were cloned into the transcription vector pGEM-T Easy using the pGEM-T Easy Vector System I (Promega). The pGEM-T Easy vector systems are convenient systems for the cloning of PCR products. The vector is prepared by cutting with *Eco*RV and adding a 3' terminal thymidine (T) to both ends. The presence of these 3'-T overhangs at the insertion site enhances the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and by providing a compatible overhang for PCR products generated by certain thermostable polymerases such as Taq polymerase. These thermostable polymerases add a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of the amplified fragments.

The PGEM-T Easy vector is a high copy number vector that contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galatosidase. Non-recombinant plasmids produce a functional α-peptide which leads to production of a functional β-galatosidase. Insertional inactivation of the α-peptide results in no β-galatosidase activity and these recombinant clones can be directly identified by colour screening on indicator plates containing IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indoyl-β-D galactopyranoside). Therefore bacterial colonies harbouring recombinant clones are white, whereas bacterial colonies harbouring non-recombinant plasmids are blue.

The following equation was used to calculate the amount of insert DNA to include in the ligation reaction.

$$\frac{\text{X ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{Y ng insert DNA}$$

For each insert DNA, ligation reactions with vector:insert ratios of 1:1, and 1:3 were performed. Each reaction was composed of 1X Rapid Ligation Buffer (30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 5% polyethylene glycol (50 ng), 3 Weiss units of T4 DNA ligase, made up to a total volume of 20 µl with nuclease free H_20. Control ligations of the vector alone, and the insert alone, with all the ligation reaction components were also included. Ligation reactions were left at 4^0C overnight.

2.3.5 Transformation of competent *E.coli* JM109 cells with plasmid DNA

The ligation of fragments with a single-base overhang can be inefficient, so *E.coli* JM109 high efficiency competent cells (Promega) were used to obtain a reasonable number of colonies. JM109 cells are also compatible with blue/white colour screening and standard Ampicillin selection. Each ligation was transformed into JM109 cells, followed by mini-scale preparation of plasmid DNA. Frozen competent JM109 cells were thawed on ice. Once the cells had just thawed, 50 µl of cells were added to a 1.5 ml eppendorf. Two microlitres of each ligation reaction were added to the cells, mixed and placed on ice for 20 min. The cells were heat shocked at 42^0C for 45-50s, followed by immediately returning to ice for 2 min. Nine hundred and fifty microlitres of room temp Luria broth (LB) (Sigma) was added to the tube and incubated in a shaking incubator at 37^0C for 1.5 hr. One hundred microlitres of the transformed cells were plated out onto LB agar plates supplemented with 50 µg/ml Ampicillin (Sigma), 0.5 mM IPTG (Promega), and 80 µg/ml X-Gal (Promega) to allow the selection of the recombinant clones (white colonies).

2.3.6 Small-scale preparations of plasmid DNA

After overnight incubation at 37^0C, the white putative recombinant clones were picked and inoculated into 5 ml LB containing 50 µg/ml Ampicillin, and incubated in a shaking incubator at 37^0C overnight. The selected clones were also streaked onto LB agar plates supplemented with 50 µg/ml Ampicillin and stored at 4^0C. Glycerol stocks were made by adding 200 µl glycerol (Sigma) to 800 µl of the overnight bacterial suspension, vortexed and snap frozen in an methanol/dry ice bath and stored at -70^0C. The remainder of the overnight bacterial suspension were used for plasmid DNA purification.

Plasmid purification was performed using the Wizard *Plus* SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Five millilitres of overnight bacterial culture were pelleted by centrifugation at 10 000 *g* for 5 min, and the supernatant discarded. Two hundred and fifty microlitres of Wizard *Plus* SV Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNase A) were added to the cell pellet, followed by 250 µl of Wizard *Plus* SV Cell Lysis Solution with mixing. Ten microlitres of Alkaline Protease Solution were then added, and the tubes were incubated at room temperature for 5 min. Three hundred and fifty microlitres of Wizard *Plus* SV Neutralization solution were added, followed by centrifugation at 10 000 *g* for 10 min. The cleared lysate was then transferred to the Wizard *Plus* SV Minipreps Spin Column, and centrifuged at 10 000 *g* for 1 min. After discarding the flow-through, 750 µl of Wizard *Plus* SV Minipreps Column Wash solution were added to the Spin Column. The Spin Column was centrifuged twice at 10 000 *g* for 1 min discarding the flowthrough each time. The Wizard *Plus* SV Minipreps Spin Column was transferred to a fresh eppendorf and plasmid DNA was eluted in 100 µl of nuclease free H_20.

2.3.7 Screening of plasmid clones by restriction endonuclease digestion

The plasmid DNAs were digested with *Eco*RI to confirm that the clones contained HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 cloned into pGEM-T Easy. Three hundred nanograms of each plasmid DNA were digested with 1U of *Eco*RI in 1X buffer H (50 mM Tris-HCl, 0.1 M NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.5 at 37^0C) (Roche) made up to a final volume of 15 µl with nuclease free H_20. The reaction was incubated at 37^0C for 2 hrs, and then analysed on a 3% agarose gel in 1X TBE buffer as described in section 2.2.2. The concentration of the plasmid DNA was measured using the Gene Quant. The presence and orientation of the insert was verified by sequencing with M13 universal and reverse primers using an in-house sequencing service at the Windeyer Institute, UCL.

M13 Universal 5' - GTA AAA CGA CGG CCA GT - 3'
M13 Reverse 5' - GTT TTC CCA GTC ACG AC - 3'

2.3.8 Linearization of the plasmids prior to transcription

From the sequencing results, all inserts were found to be cloned into the pGEM-T Easy vector in the 5'-3' orientation. The restriction enzymes *Nco*I and *Nde*I were used to digest the plasmid DNAs in order to generate insert sequence for the production of antisense and sense RNA transcripts using *in vitro* transcription with the respective Sp6 and T7 RNA polymerase as described in section 2.3.10 (see figure 2.2).

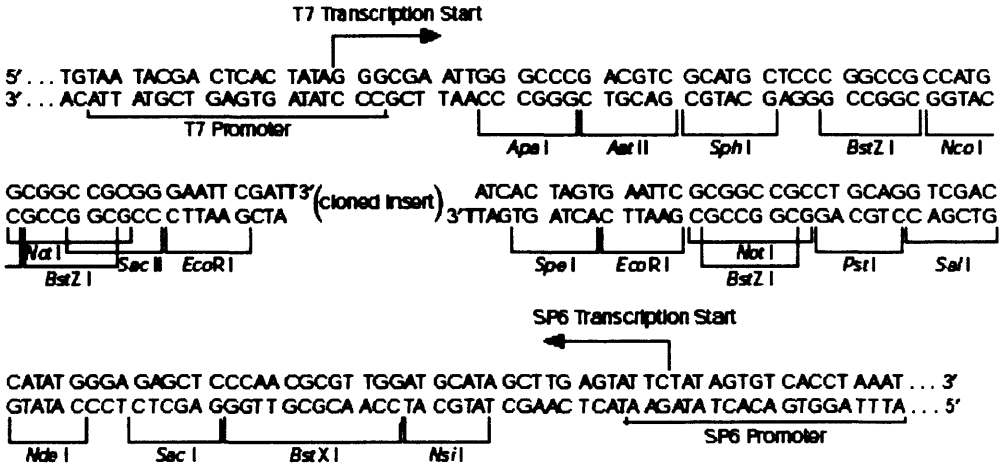


Figure 2.2 The promoter and multiple cloning sequence of the pGEM-T Easy vector. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by Sp6 RNA polymerase (www.promega.com).

Ten micrograms of each plasmid DNA were digested with 50 U restriction enzyme (*NcoI*/*NdeI*) in 1X NE buffer 4 (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 at 25^{0}C) (New England BioLabs) made up to a final volume of 50 µl with nuclease free H_20. The reaction mixture was digested at 37^{0}C for 2-3 hr. Two microlitres of the digestion reaction, together with 2 µl of the uncut plasmid were analysed on a 0.8% agarose gel in 1X TBE buffer to confirm complete digestion of the vector. The restriction digestion must be performed to completion as a small amount of undigested plasmid DNA can give rise to very long transcripts.

2.3.9 Phenol extraction and ethanol precipitation of linearized template DNA

After the restriction digest, the linearized template DNAs were purified by phenol chloroform extraction and ethanol precipitated to avoid RNase contamination. To each digest, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added, vortexed vigorously for 10 sec and centrifuged at 10 000 *g* for 15 sec. The upper, aqueous phase was transferred to a fresh 1.5 ml eppendorf, and 0.1 vol of 3 M sodium acetate buffer solution (pH 5.2) (Sigma) was added, and vortexed briefly. Two volumes of ice-cold 100% ethanol were then added to the mixture, which was vortexed, and placed in a –70^{0}C freezer for at least 30 min. After centrifuging at 10 000 *g* for 5 min the supernatant was discarded. One ml of room temp 70% ethanol were added, the tube was mixed by inverting several times, and centrifuged at 10 000 *g* for 5 min. The supernatant was discarded, and the pellet was air dried, before resuspending in 20 µl nuclease free H_20.

2.3.10 *In vitro* transcription using Sp6 and T7 RNA polymerase

As mentioned previously, the plasmid DNAs digested with *Nco*I were used to generate antisense RNA transcripts using *in vitro* transcription with Sp6 RNA polymerase, and the plasmid DNAs digested with *Nde*I were used to generate sense RNA transcripts with T7 RNA polymerase. *In vitro* transcription was performed using the riboprobe combination system-Sp6/T7 (Promega).

Each *in vitro* transcription reaction was performed in a 1.5 ml RNase-free microfuge tube (Ambion), and was composed of 1 µg linearized template DNA, 1X transcription buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 10 mM dithiotreitol (DTT), 40 U RNAsin, 20 U RNA polymerase (Sp6 or T7), and 1X DIG RNA labelling mix (1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 UTP, 0.35 mM DIG-11-UTP, pH 7.7 (20^0C) (Roche)) made up to a final volume of 20 µl with nuclease free H_20. A positive control reaction with pGEM Express Positive control template and Sp6 RNA polymerase was also included. The reaction components were added on ice, and after mixing and centrifuging briefly, *in vitro* transcription was performed for 2 hr at 37^0C. After *in vitro* transcription, any remaining template DNA was removed by adding 2 µl RQ1 RNase-free DNase (1 U/µl) (Promega) to the tube, and incubating at 37^0C for 15 min. Two microlitres of 0.2 M EDTA (pH 8.0) were then added to the tube to stop the reaction.

2.3.11 Ethanol precipitation of RNA transcripts

To each *in vitro* transcription reaction, 0.1 vol of 4 M lithium chloride (LiCl) and 2.5 vol of prechilled 100% ethanol was added to the tube, and placed in a -70^0C freezer for at least 30 min. The tubes were centrifuged at 10 000 *g* for 15 min, to pellet the precipitated RNA. The ethanol was decanted, and 50 µl of 70% ethanol was added to the tube which was inverted several times to wash the pellet. After centrifuging at 10 000 *g* for 5 min, the ethanol was decanted, and the pellet air dried. The RNA pellet was resuspended in 50 µl nuclease free H_20, and 1 µl RNase inhibitor (Promega) was added to the tube. The concentration of RNA was measured using the Gene Quant and the riboprobes were stored at -70^0C until ready for use.

2.3.12 Denaturing agarose gel preparation and electrophoresis

The RNA transcripts were analysed on a 3% denaturing agarose gel. The gel was prepared by weighing out 3 g of agarose in a 200 ml Duran bottle, and adding 10 ml of 10X morpholinopropanesulphonic acid (MOPS) buffer (0.2 M MOPS (pH 7.0), 50 mM sodium acetate, 0.5 mM EDTA (pH 8.0), and 86 ml of 0.1% diethyl pyrocarbonate (DEPC)-treated H_20. DEPC-treated H_20 was prepared by adding 1 ml DEPC to 1 litre of distilled H_20. The H_20 was shaken vigorously, and left overnight at room temp before autoclaving to inactivate the DEPC. The gel was heated in a microwave oven

until all the agarose had completely dissolved. The gel was allowed to cool to 50^0C before adding 4.8 ml formaldehyde (37% solution, Sigma). In a fume cupboard, the gel was poured into a gel-casting tray, with a gel comb, and allowed to set. Once set, the gel was placed in an electrophoresis tank containing 1X MOPS buffer. Five microlitres of each transcription reaction, together with 1 µg RNA markers 0.28-6.58 kb (Promega), were added to 15 µl RNA sample buffer (10 ml formamide (Sigma), 3.5 ml formaldehyde (37% solution), and 2 ml 10X MOPS buffer) and 2 µl RNA loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, and 1 mg/ml ethidium bromide) in a 0.5 ml tube. The tubes were placed in a heating block and denatured at 60^0C for 10 min, before loading into separate wells. The gel was run at 100V for 30 min and visualised on a UV transilluminator.

2.3.13 Spot assay to detect efficiency of digoxigenin labelling reaction

To confirm that the RNA transcripts were sufficiently labelled with DIG, a spot assay was performed. Serial dilutions of the RNA probes (10 ng-0.01 ng/µl RNA) in RNA dilution buffer (500 µl of nuclease free H_20, 300 µl 20X Saline-sodium citrate (SSC) buffer and 200 µl formaldehyde) were spotted onto Nylon Hybond N membrane (Amersham Life Sciences) and air dried for 5 min. The RNA probes were fixed onto the membrane by UV crosslinking. The membrane was placed face down on a UV transilluminator for 20 min. The membrane was then washed in buffer 1 (0.1 M Tris-HCl, 0.1 M NaCl, 2 mM $MgCl_2$, 0.5% Triton-X100, pH 7.5) followed by blocking in 2% normal sheep serum in buffer 1 for 15 min. The membrane was incubated with an anti-DIG Alkaline phosphatase (AP) Ab (Roche) diluted at 1:5000 dilution in buffer 1 with 1% normal sheep serum for 30 min. After washes in buffer 2 (0.1 M Tris-HCl, 0.1M NaCl, 50 mM $MgCl_2$, pH 9.5) for 15 min, detection was carried out using the 5-Bromo-4-chloro-3-indoylphosphate/Nitroblue tetrazolium (BCIP/NBT) AP substrate kit IV (Vector Labs). The BCIP/NBT substrate working solution was prepared according to the manufacturer's instructions. Two drops of Reagent 1, 2 drops of Reagent 2, and 2 drops of Reagent 3 were added to 5 ml 0.1 M Tris-HCl pH 9.5 mixing well each time. The membrane was incubated with BCIP/NBT substrate solution and left in the dark for approximately 1 hr or until dark purple spots appear in sufficient intensity. The reaction was stopped by rinsing in H_20 for 5 min.

2.4 Development of *in situ* hybridisation for the detection of human β-herpesvirus DNA

In situ hybridisation (ISH) is a technique that allows the detection of nucleic acid hybrid molecules (DNA:DNA, DNA:RNA or RNA:RNA) between the target nucleic acid in morphologically preserved cells or tissues, and a labelled probe containing a complementary sequence. In this section, ISH was developed for the detection of β-herpesvirus DNA using the DIG labelled riboprobes constructed in section 2.3. The ISH method was first tested on 4% paraformaldehyde fixed cell controls (section 2.4.1), and this provides a good starting point as it gives adequate morphology and sufficient permeability so that permeabilisation steps are usually not required. ISH was then carried out on formalin-fixed paraffin-embedded cell controls where optimisation of *in situ* conditions was required to achieve optimal hybridisation signals. Further optimisation of the ISH method was carried out on formalin-fixed paraffin-embedded liver and renal biopsies (sections 2.4.3 and 2.4.4). This method was subsequently used for the detection of human β-herpesvirus DNA in liver and renal biopsies from transplant patients (sections 2.5 and 2.6).

2.4.1 ISH on 4% paraformaldehyde-fixed cell suspensions

The detection of the human β-herpesviruses by ISH using DIG labelled riboprobes was tested initially on 4% paraformaldehyde-fixed cells from virus infected cultures. HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 riboprobes were tested on HCMV infected HEL/MRC-5, HHV-6 infected Molt-3, and HHV-7 infected Sup T1 cells, respectively. Non-infected HEL, Molt-3 and Sup T1 cells were used as negative controls.

2.4.1.1 Tissue pre-treatment

All pre-treatment conditions were performed under RNase free conditions when RNA probes were used. Disposable gloves were worn at all times, sterile glass and plasticware were used, and all solutions were treated with 0.1% DEPC. Surfaces were also wiped down with RNase Zap (Ambion). The paraformaldehyde-fixed cell suspensions (see section 2.1.3.1) were rehydrated through decreasing concentrations of ethanol into distilled H_20. The slides were then washed in PBS for 5 min and treated with 0.03% triton-X100 in PBS for 15 min. The slides were incubated at a range of

Proteinase K (Sigma) concentrations ranging from 0-10 µg/ml Proteinase K in Proteinase K buffer (50 mM Tris-HCL, 5 mM EDTA pH 7.6) for 30 min at 37^0C followed by PBS washes three times for 5 min. The slides were dehydrated through increasing concentrations of ethanol and air dried.

2.4.1.2 Hybridisation with DIG labelled riboprobes

Hybridisation was carried out using the GeneAmp *in situ* PCR System 1000 (Perkin Elmer). This thermal cycling machine is designed for use with the Perkin Elmer *in situ* PCR Reagent Containment System (includes the *in situ* PCR 1000 slide assembly tool, Amplicover Discs, Amplicover Clips and *in situ* PCR glass slides (Applied Biosystems)), for containing reagents over tissue specimens mounted on slides. The machine block can incubate the samples at a constant temperature. The slides were assembled onto the *in situ* PCR 1000 slide assembly unit. The Amplicover clip has stainless steel sliding grip attached at either end for gripping the slide to hold the Amplicover disc in place.

Hybridisation mix consisted of 50% formamide, 10% dextran sulphate, 1X Denhardts solution (0.02% Ficoll, 0.02% Polyvinyl pyrrolidone, 10 mg/ml RNase-Free bovine serum albumin) (Sigma), 4X SSC, 10 mM DTT, 100 µg/ml yeast tRNA (Roche), 100 µg/ml denatured and sheared Salmon sperm DNA (Sigma) and 5 ng of DIG labelled riboprobe. The salmon sperm DNA and DIG labelled riboprobe were added to the hybridisation mix immediately before hybridisation.

Fifty microlitres of the hybridisation mix were pipetted onto the centre of each cell suspension. The Amplicover clip and disc was then lowered onto the slide and by squeezing the levers on the top of the assembly tool the Amplicover clip and disc were secured onto the slide. The Amplicover disc and clip creates an airtight reaction chamber to prevent evaporation during hybridisation (or thermal cycling). The *IS* 1000 instrument was programmed for denaturation at 95^0C for 5 min followed by hybridisation at 37^0C overnight.

2.4.1.3 Post-hybridisation washes

After hybridisation, the Amplicover discs and clips were dismantled and the slides washed in solutions of decreasing salt concentrations (2X SSC twice at 37^0C for 15 min, 1X SSC twice at 37^0C for 15 min, 0.2X SSC twice at 37^0C for 30 min) to remove the excess and non-specifically bound probe and to melt away mismatched hybrids leaving only the true hybrids intact for detection.

2.4.1.4 Immunological detection of signal

The slides were washed in buffer 1 (0.1 M Tris-HCl, 0.1 M NaCl, 2 mM $MgCl_2$, 0.5% Triton X100, pH 7.5) for 5 min, and incubated in blocking solution (buffer 1 containing 2% normal sheep serum) for 30 min to remove background. DIG labelled DNA was detected with an anti-DIG AP Ab (Roche). One hundred microlitres of AP-labelled anti-DIG Ab diluted to 1:200 in buffer 1 with 1% normal sheep serum were added to the slide, and incubated in a humidified chamber at room temp for 2 hr. The slides were further washed in buffer 1 for 10 min followed by buffer 2 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 min. Detection of the DIG-labelled RNA:DNA hybrid was carried out using the BCIP/NBT AP substrate kit IV as previously described in section 2.2.13. A precipitating dark purple reaction product (NBT Formazan) is formed at the site of DIG-labelled hybrid. The reaction was stopped by washing the slides in distilled H_20 for 5 min. The slides were subsequently air dried and mounted in UV Loctite 358 adhesive.

2.4.2 ISH on formalin-fixed paraffin-embedded cell suspensions

ISH using DIG labelled riboprobes was also tested on formalin-fixed paraffin-embedded cells from cultures infected with the respective virus. HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 riboprobes were tested on HCMV infected HEL, HHV-6 infected Molt-3 and HHV-7 infected Sup T1 cells respectively. Non-infected HEL, Molt-3 and Sup T1 cells were again used as negative controls. ISH was carried out in the same way as that for paraformaldehyde-fixed cell suspensions (see section 2.3.1), except that slides were initially deparaffinized in xylene twice for 15 min at room temp.

2.4.2.1 Optimisation of protease concentration

For formalin-fixed paraffin-embedded cell suspensions, a range of Proteinase K concentrations ranging from 0-100 µg/ml Proteinase K in Proteinase K buffer were tested. Incubation was carried out at 37^0C for 30 min.

2.4.2.2 Optimisation of riboprobe concentration

Once the optimal concentration of proteinase K had been determined, the concentration of DIG labelled riboprobe was increased to see whether the hybridisation signal could be improved. A range of DIG labelled riboprobe concentrations (5 ng, 7.5 ng and 10 ng) in 50 µl of hybridisation mix was tested. Hybridisations were performed at 37^0C overnight.

2.4.2.3 Optimisation of salmon sperm DNA and yeast tRNA concentrations

Once the optimal concentration of DIG labelled riboprobe was determined, a range of concentrations of salmon sperm DNA and yeast tRNA (0, 100 µg/ml, and 1 mg/ml) in the hybridisation mix was tested. The incorporation of these nucleic acids can help reduce non-specific nucleic acid interactions. Hybridisations were performed at 37^0C overnight.

2.4.3 ISH on liver biopsies

A liver biopsy which stained positive for HCMV late protein 47-55 kDa by IHC (and independently shown to be positive for HCMV p52 protein by IHC conducted by the Histopathology Service at the Royal Free Hospital) was used to optimise ISH conditions for the detection of HCMV DNA in liver biopsy tissue using DIG labelled HCMV gB riboprobe. Positive and negative controls consisting of formalin-fixed paraffin-embedded HCMV infected and non-infected MRC-5 cells were also included.

2.4.3.1 Optimisation of protease concentration

As mentioned previously the amount of proteolytic treatment varies for different types of tissue and must be empirically determined. A range of Proteinase K concentrations ranging from 2.5-15 µg/ml were tested on the liver biopsies. The sections were incubated at 37^0C for 30 min

2.4.3.2 Optimisation of riboprobe concentration

Once the concentration of proteinase K had been determined, hybridisations were performed using increasing riboprobe concentration (10 and 20 ng probe per hybridisation reaction) to determine whether hybridisation signal could be improved. Hybridisations were performed at 37^0C overnight.

2.4.3.3 Methods to reduce endogenous alkaline phosphatase

To help reduce non-specific background in the tissue sections caused by endogenous alkaline phosphatase, two methods were tested. One method involved treating the sections in cold 20% acetic acid for 45 sec after proteinase K treatment and PBS washes. Slides were then washed in distilled H_20 for 5 min, dehydrated and air dried. The second method involved adding levamisole solution (Vector labs) to the BCIP/NBT AP substrate solution. The sections were then incubated in the substrate solution overnight in the dark as before. One drop of levamisole solution was added per 5 ml of substrate solution.

2.4.3.4 Modified ISH with recommendations from Orion Molecular Services, Manchester.

ISH using recommendations from Orion Molecular Services, Manchester, were carried out to further improve hybridisation signals and reduce background levels. The slides were deparaffinized, and rehydrated to distilled H_20. The slides were then immersed in 0.2 M HCl for 20 min. An acid pre-treatment step was included as acids denature basic proteins and in combination with proteolytic treatments can facilitate their removal, thus making the target more accessible. The slides were then treated with 0.01% Triton-X100 in PBS for 90 sec, followed by incubation with 7.5 µg/ml Proteinase K for 30 min at 37^0C. After PBS washes three times for 5 min the sections were post-fixed in 0.4% paraformaldehyde in PBS for 20 min and washed in distilled H_20 for 5 min. Sections were then treated in cold 20% acetic acid for 45 sec and washed in distilled H_20 for 5 min. The slides were dehydrated and air dried.

Hybridisation with a range of concentrations of riboprobe (50 ng, 100 ng and 200 ng) were tested. The hybridisation mix was vortexed briefly and heated at 60^0C for 10 min in a waterbath to help remove any tertiary structures, and then immediately placed on ice. The hybridisation mix was then warmed to 37^0C before pipetting 50 µl onto each

section. The slides were denatured at 95^{0}C for 5 min followed by hybridisation at 42^{0}C overnight.

Post-hybridisation washes consisted of Wash buffer 1 (0.5X SSC, 1 mM EDTA) for 20 min at 42^{0}C, Wash buffer 2 (0.15 M NaCl, 5 mM Tris-HCl (pH7.4), 0.5 mM EDTA, 50% formamide) for 10 min at 42^{0}C, and Wash buffer 3 (0.5X SSC) for 20 min at 50^{0}C and wash buffer 3 for 5 min at room temp. Slides were placed in buffer 1 for 5 min, and incubated in blocking solution (buffer 1 containing 2% normal sheep serum) for 30 min. Anti-DIG AP Ab diluted to 1:200 in buffer 1 with 1% normal sheep serum was incubated for 2 hr in a humidified chamber. Slides were washed in buffer 1 for 5 min, and then in buffer 2 for 5 min. DIG-labelled DNA was detected using NBT/BCIP AP substrate kit IV (Vector labs). The reaction was stopped by washing in distilled H_20 for 5 min. The slides were then air dried and mounted with UV Loctite 358 adhesive.

2.4.4 ISH on renal biopsies

Using the same optimised ISH conditions for the liver biopsies (see section 2.4.3), 10 formalin-fixed paraffin-embedded renal biopsies taken from transplant patients suspected of graft dysfunction were initially tested for HCMV gB DNA. To determine the concentration of protease required for an optimal hybridisation signal in renal biopsy tissue, a sample which tested positive for HCMV gB DNA was used to test a range of Proteinase K concentrations (2.5-15 μg/ml) incubated at 37^{0}C for 30 min, and comparing hybridisation signals. Positive and negative controls consisting of formalin-fixed paraffin-embedded HCMV infected and non-infected MRC-5 cells were also included.

2.4.5 Testing the specificity of HCMV, HHV-6 and HHV-7 DIG labelled riboprobes

Once the ISH method had been determined, each of the human β-herpesvirus specific riboprobes were tested against formalin-fixed paraffin-embedded cells infected with respective virus, and to each of the other human β-herpesviruses to test for cross-reactivity.

2.4.6 Testing the sensitivity of ISH with a human β-globin riboprobe

The sensitivity of ISH was tested using a β-globin riboprobe. Human β-globin DNA is present as 2 copies per cell, therefore detection of hybridisation signals in human cell suspensions using the sense probe (for the detection of DNA and not mRNA) will help determine the level of sensitivity of this method. Section 2.4.6.1-2.4.6.2 describes the construction of the human β-globin riboprobe, and section 2.4.6.3 describes ISH with this probe.

2.4.6.1 Construction of the human β-globin probe for determining the sensitivity of ISH

A human β-globin riboprobe was constructed in the same way as described in section 2.3. A 110 bp human β-globin PCR product was amplified using primers PCO3 5' ACA CAA CTG TGT TCA CTA GC 3' and PC04 5' CAA CTT CAT CCA CGT TCA CC 3' (Saiki *et al.*, 1988). PCR cycling conditions are shown below.

Hot start	95^{0}C for 15 min	}	1 cycle
Denaturation	94^{0}C for 30 sec		
Annealing	50^{0}C for 30 sec		39 cycles
Extension	72^{0}C for 30 sec		
Final extension	72^{0}C for 10 min	}	1 cycle.

The resulting PCR product was detected by electrophoresis on a 3% (w/v) agarose gel as described in section 2.3.2. The PCR product was then purified and cloned into the pGEM-T Easy vector (see section 2.3.3-2.3.7). Sequencing results showed that the 110 bp fragment of the human β-globin gene was cloned in the 3'-5' orientation. The restriction enzymes *Nde*I and *Sph*I were used to digest the plasmid DNA (see section 2.3.8) in order to generate insert sequence for the production of antisense and sense RNA transcripts using *in vitro* transcription with T7 and Sp6 RNA polymerase, respectively. The linearized plasmid DNA was then purified by phenol extraction and ethanol precipitation as described in section 2.3.9. Plasmid DNA linearized with *Nde*I was *in vitro* transcribed with T7 RNA polymerase as described in section 2.3.10. However, *Sph*I generates 3' overhangs which can result in extraneous transcripts. Therefore the 3' overhang must be converted to a blunt end using the 3'-5' exonuclease

activity of DNA polymerase I Large (klenow) Fragment before *in vitro* transcription with Sp6 RNA polymerase.

2.4.6.2 Conversion of a 3' overhang to a blunt end

The reaction was composed of 1 µg linearized template DNA, 1X transcription buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 40 U RNAsin, DNA polymerase I Large (Klenow) Fragment (5U/µg) (Promega), 1X DNA polymerase buffer (500 mM Tris-HCl (pH 7.2 at 25^0C), 100 mM $MgSO_4$, 1 mM DTT) made up to a final volume of 10 µl with nuclease free H_20. The reaction mixture was incubated at 22^0C for 15 min, before proceeding to *in vitro* transcription. To the reaction mixture, 10 mM DTT, 20 U Sp6 RNA polymerase, 1X DIG RNA labelling mix, made up to a final volume of 20 µl with nuclease free H_20 was added. *In vitro* transcription was performed for 2 hrs at 37^0C. After *in vitro* transcription, 2 µl RQ1 RNase-free DNase (1 U/µl) was added, and incubated at 37^0C for 15 min. Two microlitres of 0.2 M EDTA (pH 8.0) were then added to the tube to stop the reaction. The RNA transcripts were then ethanol precipitated as described in section 2.3.11. The RNA transcripts were analysed in a 3% denaturing agarose gel as described in section 2.3.12 and a spot assay was performed as described in section 2.3.13.

2.4.6.3 ISH with DIG labelled human β-globin riboprobe

ISH using the β-globin sense riboprobe was tested on 4% paraformaldehyde-fixed HEL cells using the method described in section 2.4.1.1-2.4.1.4, and on formalin-fixed paraffin-embedded HEL cells, using optimized ISH conditions (section 2.4.3.4).

2.4.7 Testing the sensitivity of HHV-6 ISH on a lymphoblastoid cell line with integrated HHV-6 genome (V1-LCL)

Once the conditions of ISH were optimised, the sensitivity of ISH using HHV-6 U67 riboprobe was tested on a lymphoblastoid cell line (LCL) with integrated HHV-6 genome (V1-LCL). Using a QC-PCR, the HHV-6 load of V1-LCL was previously shown to be 26 genome copies/cell. More recently using a real-time HHV-6 Taqman assay, the viral load in this cell line was determined as 12 copies/cell (Dr Duncan Clark pers. comm.). The V1-LCL was prepared by Dr Duncan Clark, and was formalin-fixed and paraffin-embedded as described in section 2.1.3.2. Positive and negative controls

consisting of HHV-6B (Z29) infected Molt 3 and noninfected Molt 3 cells were included.

2.5 Role of the human β-herpesviruses in allograft rejection following renal transplantation

2.5.1 Renal transplant patients

This study followed on from a prospective study of 52 patients who underwent their first renal transplantation between 1st August 1993 and 31st January 1995 (Kidd *et al.*, 2000). Inclusion criteria for this present study included at least one diagnostic allograft biopsy during the 120-day post-transplant period. Fine-needle biopsies were only taken when a patient was suspected of having renal dysfunction. Patients were excluded if adequate renal tissue was not included (no biopsy performed, n = 16; biopsy too small n = 6). A total of 62 allograft biopsies from 30 renal transplant patients were available for study. In these patients, biopsies were performed at a median of 21 days (range 2 to 107) after transplantation. Two or more biopsies were available from 18 patients, with a median of 2 (range 1 to 5) biopsies.

2.5.2 Immunosuppressive therapy

Immunosuppressive therapy in the immediate post-transplant period consisted of a triple drug regimen (cyclosporine A, azathioprine, prednisolone). Episodes of histologically-identified acute rejection were treated with 0.5 or 1 gram pulse doses of methyl prednisolone, and if rejection continued a course of ATG was administered.

2.5.3 Renal biopsies

Biopsy samples were routinely fixed in 4% neutral buffered formalin and paraffin-embedded. Four μm sections were cut and mounted onto APES-coated slides (Applied Biosystems). The slides were placed in a hybridisation oven and baked at 55^0C for 48 hrs. Morphological classification of the renal allograft biopsies were conducted by Dr Michael Jarmulowicz, pathologist at the Royal Free and University College Medical School, and followed the Banff '93-95' working classification (Solez *et al.*, 1993; 1996).

2.5.4 Cell controls

Formalin-fixed paraffin-embedded cells from infected cultures of HCMV (strain AD169) infected HEL fibroblasts, HHV-6B (strain Z29) infected Molt-3 and HHV-7 (strain DC) infected SupT1 T cell lines were used as positive controls for ISH and IHC. Non-infected cell cultures were used as negative controls. Formalin-fixed paraffin-embedded human leukaemic cell line HL60, and HL60 cells treated with 10 μM etoposide (kindly provided by Dr Ranmohan Wickremasinghe, Department of Haematology, Royal Free and University College Medical School), were used as positive controls for *in situ* detection of apoptosis.

2.5.5 ISH on renal biopsies

ISH using DIG labelled riboprobes were used to test each renal biopsy for HCMV gB, HHV-6 U67 and HHV-7 U42 DNA by ISH. Ten renal biopsies positive for HCMV gB DNA were also tested for HCMV US28 DNA by ISH to confirm results. Two renal biopsies which tested negative for HCMV gB DNA were also included as negative controls. The slides were deparaffinized in xylene twice for 15 mins at room temp, followed by rehydration of the tissue in decreasing concentrations of ethanol in to distilled H_20. The slides were then immersed in 0.2 M HCl for 20 mins, and washed in distilled H_20 for 5 mins. The slides were then treated with 0.01% Triton-X100 in PBS for 90 secs, followed by incubation with 7.5 μg/ml Proteinase K in Proteinase K buffer (50 mM Tris-HCL, 5 mM EDTA pH 7.6) for 30 mins at 37^0C, and washes in PBS three times for 5 mins. Sections were post-fixed in 0.4% paraformaldehyde for 20 mins and washed in distilled H_20 for 5 mins, followed by immersion in cold 20% acetic acid for 45 secs. The slides were washed in distilled H_20 for 5 mins and dehydrated through increasing concentrations of ethanol and air dried. For each renal sample, hybridisation with HCMV gB, HHV-6 U67, and HHV-7 U42 riboprobe was carried out. Fifty nanograms of DIG labelled riboprobe were added to 50 μl hybridisation mix containing 50% formamide, 10% dextran sulfate, 1X Denhardts solution, 4X SSC, 10 mM DTT, 1 mg/ml yeast tRNA and 1 mg/ml denatured and sheared Salmon sperm DNA. A negative reagent control with hybridisation mixture, but without probe, was performed in parallel for each sample. The hybridisation mix was vortexed briefly and heated at 60^0C for 10 mins in a waterbath, and then placed on ice. Fifty microlitres of probe mixture was pipetted onto each section and denatured at 95^0C for 5 mins followed by

hybridisation at 42^{0}C overnight. Post-hybridisation washes consisted of 4X SSC for 5 mins at room temp, Wash buffer 1 (0.5X SSC, 1 mM EDTA) for 20 mins at 42^{0}C, Wash buffer 2 (0.15M NaCl, 5mM Tris-HCl (pH7.4), 0.5 mM EDTA, 50% formamide) for 10 mins at 42^{0}C, and Wash buffer 3 (0.5X SSC) for 20 mins at 50^{0}C and for 5 mins at room temp. Slides were washed in buffer 1 (0.1 M Tris-HCl, 0.1 M NaCl, 2 mM $MgCl_2$, 0.5% Triton X100, pH 7.5) for 5 mins, and incubated in blocking solution (buffer 1 containing 2% normal sheep serum) for 30 mins. Anti-DIG AP Ab was diluted to 1:200 in buffer 1 with 1% normal sheep serum was incubated for 2 hours in a humidified chamber. Slides were further washed in buffer 1 for 5 mins followed by buffer 2 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 mins. DIG-labelled RNA-DNA hybrids were detected using NBT/BCIP AP substrate kit IV (Vector labs). The reaction was stopped by washing in distilled H_20 for 5 mins. The slides were air dried, and mounted with UV Loctite 358 adhesive.

2.5.6 HCMV-specific IHC

Ten renal biopsies positive for HCMV gB DNA by ISH were tested for the presence of HCMV proteins characteristic for each temporal stage of replication using the following mAbs: MAB8131 directed to the HCMV IE protein; MAb CCH2 directed HCMV p52 (Plachter *et al.*, 1992); MAB8126 to HCMV late protein. Two immunohistochemical staining methods were used. The ABC system (Elite ABC kit, Vector Labs) as described in section 2.2.1 was used with the three HCMV MAbs, and the DAKO chemMate Envision detection system (Dako) as described in section 2.2.2 was used with two HCMV MAbs (MAB8131 and MAb CCH2). The sections were counterstained with Hematoxylin (Gill's Formula) (Vectorlabs). Sections were incubated in Hematoxylin for 1-5 mins followed by rinsing in running tap H_20 until rinse H_20 was clear. The slides were then dipped 10 times in acid rinse solution (2 ml glacial acetic acid in 98 ml 70% ethanol) followed by 10 dips in tap H_20. The slides were then incubated in bluing solution (1.5 ml ammonium hydroxide (Sigma) in 98.5 ml 70% ethanol) for 1 min followed by 10 dips in tap H_20 and dehydrated through increasing concentrations of ethanol. The specificities of these Abs were confirmed by using isotype matched control mouse mAbs (Dako). Five renal biopsies negative for HCMV gB DNA by ISH were also included as negative controls.

2.5.7 *In situ* detection of active caspase-3

To investigate whether apoptosis is associated with the presence of HCMV in renal biopsies, 10 samples positive for HCMV gB DNA by ISH were stained with an anti-active caspase-3 mAb (C92-605; Pharmingen) (Dukers *et al.*, 2002a). Caspase-3 is a cysteine protease that plays a central role in apoptosis. The presence of activated caspase-3 is a sensitive and specific marker of apoptosis (Dukers *et al.*, 2002b). The slides were deparaffinized, endogenous peroxidase was blocked, and the sections were microwaved in Citrate buffer as previously described in section 2.2.1. The sections were then incubated with 10% normal goat serum in PBS with the avidin biotin blocking kit as mentioned in section 2.2.1.4. The anti-active caspase-3 Ab was then added to the sections at a 1:500 dilution in first layer diluent and incubated overnight at 4^0C in a humidified chamber. Immunohistochemical detection was carried out, and the sections were counterstained with hematoxylin as described in section 2.5.6. For each sample, staining with diluted normal goat serum was included as a negative control. Human leukaemia cell line HL-60 cells, with and without the treatment of 10 μM etoposide, an inducer of apoptosis, was used as positive controls.

2.5.8 *In situ* detection of C4d

All renal biopsies were tested for the presence of Ab mediated rejection using a polyclonal peptide-specific anti-C4d antibody (C4dpAb; Biomedica) (Regele *et al.*, 2001). Immunohistochemical staining using the DAKO chemMate Envision detection system was performed as described in section 2.2.2. The sections were incubated with C4dpAb (4 μg/ml) in polyclonal Ab diluent (0.3% Triton X-100, 2% normal goat serum, 0.01% sodium azide in PBS) for 30 mins at room temp. For each experiment, a renal allograft biopsy positive for C4d staining, and a renal allograft biopsy negative for C4d staining was included. A liver allograft biopsy was also included as a negative control. For 20 samples which stained positive for C4d, normal rabbit IgG serum (Sigma) diluted to the same concentration as the anti-C4d Ab was included as a negative control.

2.5.9 Statistical analyses

Statistical comparison between the groups was done using the X^2 and Fisher's exact tests.

2.6 Investigation of HCMV gene expression in renal biopsies

To investigate HCMV gene expression in the renal biopsies of the transplant recipients, a DIG labelled riboprobe specific to a region of the HCMV IE-1 was constructed for ISH. The IE-1, also referred to as the major IE gene, is the first gene to be expressed following the entry of the HCMV into the cell, and requires no prior protein synthesis for its expression (LaFemina and Hayward, 1983). Since IE gene expression can occur during an abortive or productive infection, development of a HCMV IE-1 probe will be useful to detect HCMV in various states of infection.

2.6.1 Construction of HCMV immediate early (IE) riboprobes

HCMV IE riboprobe was generated by subcloning a HCMV IE-1 region of cDNA into the pGEM-T Easy vector, followed by *in vitro* transcription to generate both sense and antisense RNA transcripts. The antisense probe is a complementary strand to the mRNA, and should hybridise to a region on the target mRNA. The sense probe should not hybridise, since it is the same sequence as the target mRNA.

2.6.1.1 Extraction of RNA from HCMV infected HEL cells

HEL cells infected with HCMV for 4 hrs, and uninfected HEL cells were cultured in 75 cm^2 flasks as described in section 2.1.1, and 2.1.2. Total RNA was then extracted using TRIzol reagent (GibcoBRL). The medium was first discarded from the HEL cells, and 6 ml TRIzol was added to the cell monolayer. The cells were lysed by repetitive pipetting. The cell lysate was transferred to 1.5 ml RNase-free tubes (6 tubes of 1 ml cell lysate) and heated at 70^0C on a heating block for 10 min to facilitate dissociation of RNA from the cell debris/nucleoprotein complexes. The tubes were left to cool to room temp, and then 0.2 ml chloroform was added to each tube. The tubes were shaken vigorously for 15 sec, and left to stand at room temp for 3 min. The tubes were centrifuged at 10 000 g for 15 min. After centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. Five hundred microlitres of the aqueous phase were transferred to a fresh RNase-free tube and 0.5 ml isopropanol were added to precipitate the RNA. The tubes were left at room temp for 10 min, and then centrifuged at 10 000 g for 10 min to pellet the RNA. The supernatant was discarded, and the pellet was air dried before

resuspending in 50 µl RNase-free H_20. The tubes were incubated at 55^0C for 10 min, and stored at -80^0C. The concentration of RNA was measured using the GeneQuant.

2.6.1.2 RQ1 RNase-Free DNase treatment of RNA

The RNA sample was treated with RQ1 RNase-Free DNase (DNase I) (Promega) to remove any residual DNA. Each reaction was composed of 20 µg RNA in 1X Reaction buffer (40 mM Tris-HCl (pH 8.0), 10 $MgSO_4$, 1 mM $CaCl_2$), 20 U RQ1 RNase-Free DNase, made up to a final volume of 50 µl with nuclease free H_20. The reaction mixture was incubated at 37^0C for 30 min. One microlitre of RQ1 DNase Stop solution (20 mM EDTA, pH 8.0) was added to terminate the reaction. The tubes were incubated at 65^0C for 10 min to inactivate the DNase.

2.6.1.3 Reverse transcription (RT)-PCR

Extracted RNA was reverse transcribed for generation of cDNA template for PCR amplification. PCR amplification was carried out using HCMV IE-1 primers IEP3D 5' CCA GAC TCA GCT GAC TGT TAA CCT CCT TCC 3' and IEP2AII 5' ATG GAG TCC TCT GCC AAG AGA AAG ATG GAC 3' (Kondo *et al.*, 1994). These two primer pairs span exon 2-3 (see figure 2.3), so that amplification of potentially contaminated genomic DNA sequences would produce PCR products that are larger (263 bp) than the expected mRNA product (151 bp), as intron sequences that are excised during RNA processing would be included in the genomic DNA.

cDNA was synthesized using random hexamer primers (Perkin Elmer), and Omniscript reverse transcriptase (Promega). Two micrograms of RNA were reversed transcribed in 1X PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.5 mM of dNTPs, 10 U RNase inhibitor, 4 U Omiscript RT, and 2.5 µM random hexamer primers made up to a final volume of 20 µl in nuclease free H_20. Each reaction component was added on ice. The RT reaction was incubated at 37^0C for 1 hr, and inactivated by 95^0C for 3 min, followed by rapid cooling on ice. PCR was performed in 1X PCR buffer containing 2 mM $MgCl_2$, 200 µM each dNTP, 1.25 U of HotStar Taq DNA Polymerase and 100 nM of each primer in a volume of 45 µl made up with nuclease free H_20. Five microlitres of cDNA (0.1 µg/µl) were added to each reaction. Negative controls of HCMV infected HEL RNA with no RT step, uninfected HEL cDNA, and distilled H_20 were included.

The PCR cycling conditions previously described by Kondo *et al.* (1994) are shown below. Cycling was performed on a Hybaid DNA thermal cycler.

Hot start	95^{0}C for 15 min	} 1 cycle
Denaturation	94^{0}C for 1 min	
Annealing	62^{0}C for 1 min	30 cycles
Extension	72^{0}C for 2 min	
Final extension	72^{0}C for 10 min	} 1 cycle.

2.6.1.4 Gel preparation and electrophoresis

The resulting PCR amplicons were detected by electrophoresis on a 3% (w/v) agarose gel in 1X TBE buffer as described in section 2.3.2. Visualisation of the gel showed the presence of primer dimers, and contaminating genomic DNA products (263 bp), as well as the expected cDNA product (151 bp), therefore purification of the 151 bp product from agarose was performed, instead of direct purification from the tubes as previously described.

2.6.1.5 Purification of cDNA amplification product from agarose gel

The remaining volume of cDNA product (approximately 40 µl/tube) was run on a 3% (w/v) low-gelling point agarose gel in 1X TBE buffer. The bands were visualised under long or medium U.V light to minimise exposure of DNA to the light, and the 151 bp fragment was excised from the agarose gel with a sterile scapel blade. The excised band was then purified from the agarose gel using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Six volumes of buffer QG was added to 1 volume of agarose gel (approximately 300 µl) in a 1.5 ml eppendorf tube. The tubes were placed in a 50^{0}C waterbath until the gel slice had completely dissolved. One gel volume of isopropanol was added to the tube and mixed briefly. The gel mixture was applied to the QIAquick column and centrifuged at 10 000 *g* for 1 min. The flow-through was discarded, and 0.5 ml of Buffer QG was added. After centrifugation at 10 000 *g* for 1 min, 0.75 ml of Buffer PE was added. The QIAquick column was centrifuged twice at 10 000 *g* for 1 min, discarding the flow-through each time. DNA was eluted by adding 50 µl of nuclease-free H_20 to the QIAquick column and centrifuging at 10 000 *g* for 1 min.

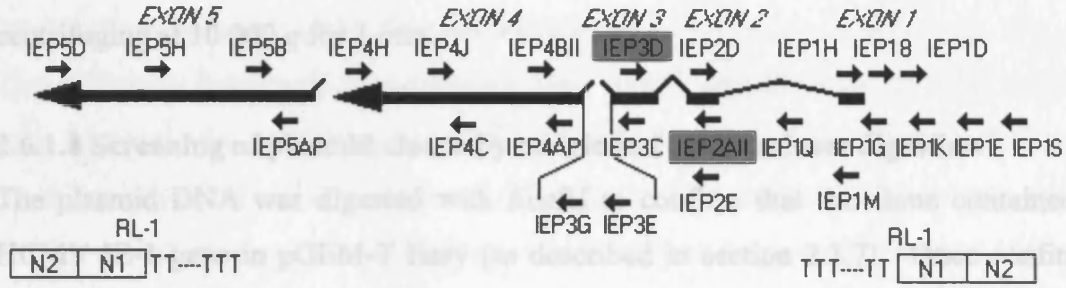


Figure 2.3. Primers used for the analysis of sense and antisense IE-1/IE-2 region transcripts (adapted from Kondo and Mocarski, 1995). Highlighted primers were used for the generation of HCMV IE-1 riboprobe.

2.6.1.6 Cloning of HCMV IE-1 into the pGEM-T Easy vector

After PCR purification, the HCMV IE-1 DNA was ligated to pGEM-T Easy as described in sections 2.3.4, and transformed into competent *E.coli* JM109 cells as described in sections 2.3.5.

2.6.1.7 Small-scale preparations of plasmid DNA

The recombinant clones were selected and purified with the QIAprep Spin Miniprep kit (Qiagen) according to the maufacturer's instructions, instead of the Wizard *Plus* SV Minipreps DNA Purification System (Promega) as described in section 2.3.6, as it was recommended that the QIAprep Spin Miniprep kit gives better quality and yield of plasmid DNA.

Five mililitres of overnight bacterial culture was pelleted by centrifuging at 10 000 *g* for 5 min. The supernatant was discarded, and the bacterial pellet was resuspended in 250 µl of Buffer P1. Two hundred and fifty microlitres of Buffer P2 was added, followed by addition of 350 µl of Buffer N3. After centrifuging at 10 000 *g* for 5 min, the supernatant was transferred to a QIAprep column in a 2 ml collection tube, and centrifuged at 10 000 *g* for 1 min. The flow-through was discarded, and 0.5 ml of

Buffer PB was added and centrifuged for 1 min, followed by a second wash in 0.75 ml of Buffer PE and centrifuging again for 1 min, discarding the flow-through each time. DNA was eluted by adding 50 µl of nuclease-free H_20 to the QIAprep column and centrifuging at 10 000 *g* for 1 min.

2.6.1.8 Screening of plasmid clones by restriction endonuclease digestion

The plasmid DNA was digested with *Eco*RI to confirm that the clone contained the HCMV IE-1 gene in pGEM-T Easy (as described in section 2.3.7). Once confirmed, the plasmid was sequenced in house at the Windeyer Institute, UCL. Sequencing results showed that the HCMV IE-1 gene was cloned in the 3'-5' orientation, and so the plasmid was linearized with *Nde*I and *Nco*I as described in section 2.3.8. After phenol chloroform extraction and ethanol precipitation of the linearized plasmid as described in section 2.3.9, *in vitro* transcription with T7 and Sp6 RNA polymerase on *Nde*I and *Nco*I linearized plasmids was carried out to generate antisense and sense RNA transcripts respectively, as described in section 2.3.10.

2.6.2 Optimisation of ISH for the detection of HCMV IE mRNA

Using previously optimised conditions for HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 riboprobes, ISH using the HCMV IE-1 antisense riboprobe was carried out on formalin-fixed paraffin-embedded MRC-5 cells infected with HCMV (AD169) for 4 hr for the detection of HCMV IE mRNA. However, background was a problem so re-optimisation of ISH conditions was performed.

2.6.2.1 Reducing background by acetylation in 0.25% acetic anhydride in 0.1 M triethanolamine

To help reduce tissue stickiness which can cause background, the sections were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. The slides were deparaffinized, rehydrated and treated with 0.2 M HCl for 20 min, followed by 0.01% Triton-X100 in PBS for 90 sec. After incubation with 5 µg/ml Proteinase K in Proteinase K buffer for 30 min at 37^0C, and washes in PBS three times for 5 min, the sections were post-fixed in 0.4% paraformaldehyde in PBS for 20 min. The slides were then washed in PBS for 5 min, followed by incubation in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 twice for 10 min each. Acetic anhydride was added to 0.1 M triethanolamine immediately before use. The slides were then plunged into 20% cold

acetic acid for 45 sec, followed by 5 min wash in distilled H_20. The sections were then dehydrated, and air dried.

2.6.2.2 Optimising hybridisation conditions

The following hybridisation conditions were tested; hybridisation at 37^{0}C overnight with 5 ng and 10 ng antisense riboprobe; hybridisation at 42^{0}C overnight with 5 ng, 10 ng, 25 ng and 50 ng antisense probe per 50 µl hybridisation mix. Hybridisation with the corresponding sense probe was performed in parallel and was used as a control for non-specific hybridisation.

2.6.2.3 Incorporation of RNase A digestion to remove single stranded (unbound) probe

To help reduce background, RNase A digestion was incorporated after hybridisation to remove any single stranded RNA probe. After pretreatment of sections, hybridisation was carried out at 42^{0}C overnight with 50 ng antisense riboprobe. The sections were then washed in 4X SSC for 5 min, wash buffer 1 for 20 min at 42^{0}C, and wash buffer 2 for 10 min at 42^{0}C. The sections were equilibrated in RNase A buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) for 5 min, followed by incubation in 25 µg/ml RNase A for 10, 20 and 30 min at 37^{0}C. After washes in RNAse A buffer three times for 5 min, the sections were washed in wash buffer 3 for 20 min at a range of temp (37^{0}C, 42^{0}C, 45^{0}C and 50^{0}C). Immunological detection with anti DIG AP Ab was performed as previously described.

2.6.3 Detection of HCMV IE mRNA expression in renal biopsies by ISH

Ten HCMV gB DNA positive samples were tested for HCMV mRNA expression using HCMV IE antisense riboprobe. The samples were pretreated in the same way as described in section 2.5.5. An acetic anhydride step was also incorporated after proteinase K digestion and PBS washes (see section 2.6.2.1). Hybridisation with 50 ng HCMV IE-1 antisense riboprobe was carried out at 42^{0}C overnight. For each sample, hybridisation with the HCMV IE-1 sense probe was included as a negative control. A previously tested HCMV gB DNA negative renal biopsy was included in each experimental run. After hybridisation, the sections were washed in 4X SSC for 5 min, followed by Wash buffer 1 for 20 min at 42^{0}C, Wash buffer 2 for 10 min at 42^{0}C, and

RNAse A buffer for 5 min at room temp. The sections were then incubated in 25 μg/ml RNase A for 30 min at 37^{0}C, followed by washes in RNAse A buffer three times for 5 min. A further wash in 0.5X SSC for 30 min at 42^{0}C and then for 5 min at room temp was carried out. Immunological detection of the *in situ* reaction was then carried out as described in section 2.5.5.

2.6.4 *In situ* hybridisation with a human β-actin riboprobe to determine quality of mRNA in tissues

A human β-actin antisense riboprobe (Roche) was used to determine the quality of mRNA in the renal biopsies. The β-actin riboprobe had been *in vitro* transcribed in the presence of digoxigenin-UTP. The transcript has a length of 588 bases, of which 550 bases are complementary to the 5' region of human-β actin mRNA (Ponte *et al.*, 1984).

2.6.4.1 Detection of β-actin mRNA on 4% paraformaldehyde fixed HEL cells

ISH with the human β-actin probe was carried out initially on 4% paraformaldehyde-fixed HEL cells using the same method as described in section 2.3.1. A negative control incorporating RNAse treatment to degrade cellular RNA was also included to check that hybridisation signals were mRNA specific. RNase was incorporated after proteinase K treatment and PBS washes. The sections were treated with 1 mg/ml RNase A in DNase buffer at 37^{0}C for 1 hr. After 5 min wash in PBS, the sections were postfixed in cold 0.4% paraformaldehyde in PBS for 20 mins.

2.6.4.2 Detection of β-actin mRNA on formalin-fixed paraffin-embedded cells

ISH using the β-actin probe was also tested on formalin-fixed paraffin-embedded HEL cells as described in section 2.4.4. Hybridisation using a range of concentrations of probe (50 ng, 25 ng, and 5 ng per reaction) were tested. Controls consisting of cells treated with DNAse I (0.1U/μl) for 1 hr at 37^{0}C, and cells treated with RNase A (1mg/ml and 0.1 mg/ml) for 1 hr at 37^{0}C after proteinase K digestion and PBS washes were also run in parallel. In addition, 10 μl RNase inhibitor (40U/ μl) was added to the hybridisation mix to help further inhibit any RNases.

2.6.4.3 Detection of β-actin mRNA on formalin-fixed paraffin-embedded renal biopsies

Five renal biopsies were initially tested with β-actin riboprobe using the same ISH method as described in section 2.4.4. Results were difficult to interpret as background staining was very high. To help reduce background levels an acetic anhydride step was incorporated during pre-treatment of sections (see section 2.6.2.1). Hybridisation using a lower concentration of probe (5 ng per reaction) was also carried out at 42^0C overnight. Ten renal biopsies (5 HCMV gB DNA positive and 5 HCMV gB DNA negative renal biopsies) were again tested. For each sample, an RNAse treated sample was run in parallel to ensure hybridisation signals were mRNA specific.

2.7 Role of human β-herpesviruses in allograft rejection following liver transplantation

2.7.1 Liver transplant patients

This study utilised a sub-section of 60 patients enrolled on a prospective study who underwent their first liver transplantation between 1st August 1993 to 11th October 1995 (Griffiths *et al.*, 1999a). Protocol liver biopsies of the donor liver were obtained at the time of transplantation; on days 5, 10, 15 and 25 after transplantation; whenever liver dysfunction occurred and after antirejection treatment. Inclusion criteria for this present study was the availability of at least one fine needle biopsy taken near the time of PCR positivity in blood for any of the human β-herpesviruses (median –2.5, range -33 to +8 days) during the 120-day post-transplant period. Those patients who had more than one PCR positive blood sample for the same human β-herpesviruses, biopsies taken nearest the time of maximum virus load were selected. In addition, consecutive liver biopsies were taken for 4 patients. Patients were excluded if adequate tissue was not included (biopsy too small n = 17). A total of 54 allograft biopsies from 30 liver transplant patients were available for study.

2.7.2 Liver biopsies

Biopsy samples were routinely fixed in 4% neutral-buffered formalin and paraffin-embedded. Four μm sections were cut and mounted onto APES-coated slides. The slides were placed in a hybridisation oven and baked at 55^0C for 48 hrs.

2.7.3 Cell controls

Formalin fixed paraffin embedded cells from infected cultures of HCMV (strain AD169) infected Human Embryonic lung (HEL) fibroblasts, HHV-6B (strain Z29) infected Molt-3 and HHV-7 (strain DC) infected SupT1 T cell lines were used as positive controls for ISH and IHC. Non-infected cell cultures were used as negative controls.

2.7.4 IHC on liver biopsies

IHC was performed using human β-herpesvirus MAbs specific for the late proteins of these viruses; MAB 8126 (Chemicon International) directed to the HCMV late protein of MW 47-55 kDa; MAb directed against HHV-6 glycoprotein gp116/64/54 (Advanced Biotechnologies), and MAb 5E1 directed against HHV-7 phosphoprotein 85 (pp85) (kindly provided by Dr Gabriella Campadelli-Fiume). The specificities of these Abs were confirmed by testing isotype matched control mouse mAbs against human IgG (1gG1, X931; IgG2a, X943; IgG2b, X944; Dako) in positive cases. All immunohistochemical stainings were performed using the ABC system as described in section 2.2.1.

2.7.5 ISH on liver biopsies

ISH using DIG labelled riboprobes were used to test each liver biopsy for HCMV gB, HHV-6 U67 and HHV-7 U42 DNA, respectively. ISH was performed as that described in section 2.4.5. A negative reagent control with hybridisation mixture, but without probe, was also performed in parallel for each sample.

Ten liver biopsies which tested positive for HCMV gB DNA by ISH were also tested for HCMV US28 DNA to compare hybridisation signals with HCMV gB. Five HCMV gB DNA negative biopsies were included as negative controls.

2.7.6 Statistical analyses

Statistical comparison between the groups was done using the X^2 and Fisher's exact tests.

2.8 HHV-6 Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) was conducted in collaboration with Dr Elisabeth Nacheva, Dr Diana Brazma, and Dr Paul Sinclair, Department of Haematology, Royal Free and University College Medical School. Three individuals were identified with abnormally high HHV-6 loads, a characteristic of viral integration into host cell chromosomes; a healthy individual (V1) with consistently high HHV-6 viral loads in the PBMCs which remained constant over a 10-month period (Clark *et al.*, 1996), a stem cell donor with a viral load of $8x10^6$ HHV-6 genomes/ml of blood, and the sibling recipient who was initially HHV-6 PCR negative following transplantation who subsequently became positive at the time of engraftment and developed an abnormally high viral load which remained constant. The PBMCs of VI and the stem cell donor were immortalized by infection with EBV to give rise to LCLs (conducted by Dr Duncan Clark. (Silveira *et al.*, 2002). Attempts to generate an LCL from the stem cell recipient were unsuccessful as B cell counts were very low in peripheral blood. FISH was developed using HHV-6 specific probes to look for integration of HHV-6 viral sequences into human chromosomes of these individuals.

2.8.1 Development of FISH using direct labelling of a HHV-6 specific plasmid probe

2.8.1.1 Direct labelling of a HHV-6 plasmid probe with SpectrumOrange by nick translation

The HHV-6 probe is a plasmid (pZVH14) (kindly provided by Professor Ruth Jarrett, University of Glasgow, UK) (Josephs *et al.*, 1986) containing an 8.7 kb *Hind*III fragment of HHV-6 DNA. This probe was labelled with SpectrumOrange using the Vysis nick translation kit (Abbot Labs). The following components were added to a 0.5 ml microfuge tube on ice: 1 µg extracted plasmid DNA, nuclease free H_20 made up to a volume of 17.5 µl, 2.5 µl SpectrumOrange (0.2 mM), 5 µl dTT (0.1 mM), 10 µl dNTP mix (0.3 mM dCTP, 0.3 mM dATP, 0.3 mM dGTP), 5 µl 10X nick translation buffer (500 mM Tris-HCl, pH 7.2, 100 mM $MgS0_4$, 1 mM DTT), and 10 µl nick translation enzyme (DNA pol I, DNase I in 50% glycerol, 50 mM Tris-HCl, pH 7.2, 10 mM MgS04, 0.1 mM DDT, 0.5 mg/ml nuclease-free BSA (bovine serum albumin). The tube was then vortexed, and centrifuged briefly, before incubating at 15^0C for 14 hr. The reaction was stopped by heating at 70^0C for 10 min, and then chilled to 4^0C

2.8.1.2 Ethanol precipitation of the probe

The following components were added to a 0.5 ml microfuge tube: 5 µl probe (100 ng), 10 µl Human Cot-1 DNA (10 µg, Invitrogen), 1.5 µl sodium acetate (pH 5.2), and 2.2 vols 100 % ice cold ethanol. The tube was then vortexed briefly, and placed at –80 ^{0}C for at least 30 min. After centrifuging at 12 000 *g* for 15 min, the ethanol was decanted, and the pellet was resuspended in 30 µl of prewarmed (37^{0}C) hybridisation buffer for 30 min or until pellet fully dissolved. The probe was stored at –80^{0}C until ready for use.

2.8.1.3 Cell line preparation

Cells were cultured by Dr Duncan Clark. The optimal seeding density for LCL is 1x10^{6}/ml.

2.8.1.4 Harvesting cells and preparation of slides

Cells from the tissue culture flasks were transferred to a falcon tube. One hundred microlitres of colcemid (10 µg/ml, Invitrogen) were added to 10 ml cells (LCL), and incubated at 37^{0}C for 60 min. The cells were then centrifuged at 400 *g* for 10 min, and the supernatant was removed. The cell pellet was then resuspended in 10 ml of hypotonic KCl prewarmed to 37^{0}C, and incubated at room temp for 20 min. The cells were then centrifuged at 400 *g* for 10 min, and the supernatant was removed. The cell pellet was loosened by gentle tapping of the tube, and with simultaneous mixing approximately 1 ml of ice cold fixative (3 parts of methanol and 1 part of acetic acid) was added drop wise to the cells. A further 7 ml of fixative was then added to the cells and mixed well by inverting the tube several times. The tube was then centrifuged at 400 *g* for 10 min, and the supernatant removed. Again the cell pellet was loosened and 5 ml of fixative was added to the cells and mixed. After centrifuging for a further 10 min, the supernatant was removed and the fixative was added until the cell suspension was slightly cloudy. Using a glass Pasteur pipette, 2 drops of the cell suspension were dropped onto a cold wet slide. The slide was tilted to allow the metaphase chromosomes to run along the length of the slide. The slide was then air dried overnight at room temp.

2.8.1.5 Staining of metaphase chromosomes

The slides were incubated in 2X SSC at 37^{0}C for 1 hr. Twenty microlitres of RNAse working solution (10 mg/ml in 2X SSC) were then applied to the target region, and covered with a coverslip. The slides were then incubated in a well-humidified chamber at 37^{0}C for 30 min. Any remaining enzyme was removed by rinsing the slides in PBS. The slides were then incubated in 0.005% pepsin in 10 mM HCl at room temp for 10 min, followed by incubation in 10 mM $MgCl_2$ in PBS at room temp for 5 min. The slides were dehydrated by immersing in increasing concentrations of ice-cold ethanol (70%, 80% and 100%) for 2 min each, and then air dried at room temp. After denaturing the slides in prewarmed 70% formamide in 2X SSC at 72^{0}C for 2 min, the slides were dehydrated by immersing in increasing concentrations of ice-cold ethanol and air dried at room temp.

2.8.1.6 Hybridisation of probes to the metaphase chromosomes

The probe solution (prepared in section 2.8.1.2) was denatured for 5 min in a 72-73^{0}C waterbath, followed by annealing at 37^{0}C for at least 1 hr. Ten microlitres of the probe solution were then added to each slide. The slides were covered with a coverslip and sealed with rubber cement glue. The slides were then incubated in a well-humidified chamber at 37^{0}C overnight.

2.8.1.7 Washing and counterstaining

The seal and coverslips were removed from the slides. The slides were then placed in a prewarmed coplin jar with 0.4X SSC/0.3% IGEPAL at 72-73^{0}C for 5 min, followed by 2X SSC/0.1% IGEPAL washes twice at room temp for 2 min. Thirty microlitres of propidium iodide and diamino-2-phenylindole (DAPI) diluted 1:100 in Vectashield mounting medium (Vector labs) was then added to each slide to counterstain the chromosomes. The slides were covered with a coverslip, and the edges were sealed with clear nail varnish. The slides were placed in a box and left to mature at 4^{0}C overnight.

2.8.1.8 FISH analysis

Fluorescence imaging of metaphase chromosomes utilised a SmartCapture FISH station (Digital Scientific).

2.8.2 Development of FISH using indirectly labelled HHV-6 plasmid probe

The HHV-6 plasmid probe (pZVH14) was indirectly labelled using biotin-16-dUTP (Roche) by nick translation using the same method as described in section 2.8.1.1. SpectrumOrange (0.2mM) was replaced with biotin dUTP (0.2mM). The probe was then ethanol precipitated as described in section 2.8.1.2. Metaphase chromosomes were prepared using cells prepared in section 2.8.1.4. Staining of metaphase chromosomes was carried out as described in section 2.8.1.5. The probe solution was then denatured and annealed as described in section 2.8.1.6. Hybridisation of metaphase chromosomes was carried out in a well-humidified chamber at 37^0C for 24 and 48 hr. The seal and coverslips were then removed from the slides. The slides were then placed in a prewarmed coplin jar with 0.4X SSC/0.3% IGEPAL at 72-73^0C for 2 min, followed by 2X SSC/0.1% IGEPAL at room temp for 2 min. After gently tapping the slides to remove any excess liquid, 100 µl of 3% BSA in 4XSSCT (0.05% triton in 4XSSC) was added to each slide, covered with parafilm and incubated at room temp for 20 min to block non-specific hybridisation signals. The slides were then washed in 4XSSCT for 5 min at room temp, and 30 µl of fluorescein isothiocynate (FITC) labelled avidin was added to each slide. The slides were covered with parafilm and incubated in a humidified chamber at 37^0C for 15 min. After washing the slides in 2X SSC/0.1% IGEPAL for 2 min at room temp, 30 µl of DAPI counterstain was added to each slide. The slides were covered with a coverslip and the edges were sealed with a clear nail varnish. The slides were placed in a box and left to mature in the dark at 4^0C overnight.

2.8.3 Development of FISH using a cocktail of HHV-6 specific plasmids

This work was done in collaboration with Dr Nacheva, Dr Brazma, and Dr Hoe Nam Leong (Centre for Virology). A cocktail of eight plasmids containing between 9 and 16kb inserts of the HHV-6 genome (non-overlapping) (see table 2.2) were used as probes for FISH (7 plasmids were kindly provided by Dr Scott Schmid, Centers for Disease Control, Atlanta (Lindquester *et al.*, 1996) and PZVH14 (see fig 2.4). These HHV-6 plasmids were combined and labelled with SpectrumGreen dUTP (Roche) using nick translation as described in section 2.8.1.1. FISH was performed using the same methods as described in sections 2.8.1.

HHV-6 plasmid probe	Z29 base position	size (kb)
BamHI C (pH6Z-102)	131777-144687	12.9
ClaI B (pH6Z-604)	110637-126162	15.5
ClaI D (pH6Z-606)	12977-22098	9.1
HindIII C (pH6Z-204)	137525-149857	12.3
HindIII E (pH6Z-205)	69994-78900	8.9
SalI D (pH6Z-816)	81792-93695	11.9
SalI G (pH6Z-802)	99666-108679	9
pZHV14	43854-52568	8.7

Table 2.2 HHV-6 specific plasmid probes for FISH

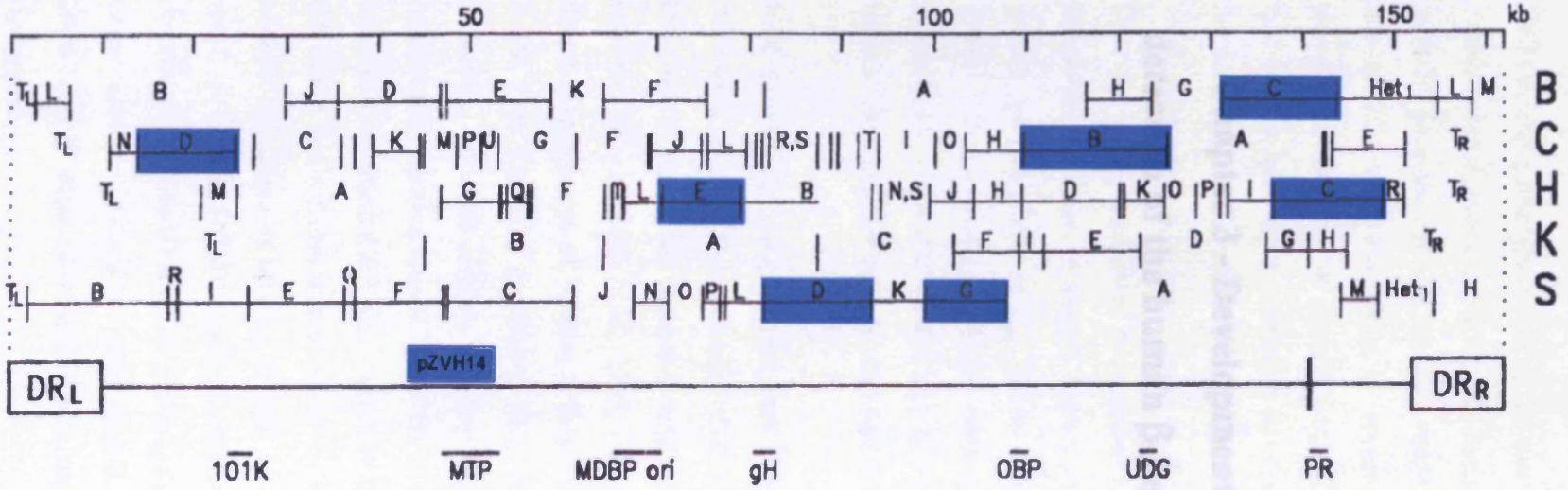


Figure 2.4 HHV-6 (Z29) restriction map showing genomic locations of cloned fragments (adapted from Lindquester *et al.*, 1996). Highlighted areas show HHV-6 plasmids used for HHV-6 FISH. All plasmids used were non-overlapping except for BamHI C (pH6Z-102) and HindIII C (pH6Z-204). Abbreviations: *101K* 101 KDa major antigenic protein; *MTP* major tegument protein ; *MDBP* major DNA-binding protein; *ori* origin of DNA replication; *gH* glycoprotein H ; *OBP* origin-binding protein ; *UDG* uracil-DNA glycosylase; *PR* parvovirus rep gene homologue; *B Bam*HI; *C Cla*I; *H Hind*III; *K Kpn*I; *S Sal*I; *TL* Term L; *TR* Term R.

Chapter 3 - Development of *in situ* techniques for the detection of the human β-herpesviruses in biopsy material.

3.1 Introduction

HCMV, the prototype member of the β-herpesvirinae, has a major impact on the immunocompromised including the recipients of solid organs and bone marrow as well as AIDS patients. HCMV causes a range of disease manifestations, ranging from a mild non-specific viral syndrome to severe end organ disease (hepatitis, pneumonitis, encephalitis, retinitis or gastrointestinal ulceration). Among SOT recipients, several risk factors have been identified for the potential development of HCMV end-organ disease. These include primary infection (Betts *et al.*, 1977), viraemia (Kidd *et al.*, 1993) and peak viral load (Cope *et al.*, 1997a; 1997b; Hassan-Walker *et al.*, 1999). Over the years, a series of prospective studies using QC-PCR have been employed to define the quantitative natural history of HCMV infection in different post-transplant patient groups (Cope *et al.*, 1997a; 1997b; Hassan-Walker *et al.*, 1999; Gor *et al.*, 1998). These studies provide evidence that HCMV load is actually the major determinant of HCMV disease and that primary infection, and viraemia are prognostic because they identify patients destined to have high viral loads.

Modern tests including DEAFF, shell vial, antigenemia and qualitative PCR assays have been used for routine monitoring of HCMV in blood. Studies have shown that PCR or antigenemia assays are important in providing prognostic information in post-transplant patients (Meyer-Konig *et al.*, 1995; Caballero *et al.*, 1997; Manez *et al.*, 1996). In our centre (Department of Virology, Royal Free and University College Medical School, Royal Free Campus) qualitative PCR is used for the routine monitoring of HCMV DNaemia, and pre-emptive therapy is recommended whenever a patient has two consecutive positive bloods (Griffiths *et al.*, 1999b). Recently, a quantitative Taqman assay has superceded the quantitative PCR assay. Whilst the measurement of HCMV in the blood is a reliable indication of the degree of viraemic dissemination, it does not address the questions relating to viral involvement in disease pathogenesis at the tissue level. At present HCMV end-organ involvement is routinely diagnosed by biopsy, with the histologic identification of cytomegalic inclusion bodies in routine H and E stained tissue biopsies. This is a highly specific, but rather insensitive method (Mattes *et al.*, 2000). As a result more sensitive *in situ* methods including IHC and ISH have been developed.

The detection of HCMV Ags with MAbs using IHC has provided a rapid and more sensitive method compared to detection of inclusion bodies for the diagnosis of HCMV infection in immunocompromised patients (Niedobitek *et al.*, 1988; Sacks and Freeman, 1984; Paya *et al.*, 1990). ISH has also been shown to be specific and more sensitive compared with typical histological criteria for the diagnosis of HCMV pneumonitis, hepatitis or encephalitis in the immunocompromised host (Myerson *et al.*, 1984; Naoumov *et al.*, 1988; Masih *et al.*, 1988; Musiani *et al.*, 1994).

In addition to direct end-organ involvement, HCMV has been associated with indirect effects including accelerated atherosclerosis, increased predisposition to other opportunistic infections, and allograft rejection in SOT patients (Rubin, 1989). Similarly, the other members of the human β-herpesviruses, HHV-6 and HHV-7 (Frenkel *et al.*, 1990; Salahuddin *et al.*, 1986), have also been implicated in disease pathogenesis in the immunocompromised. Case reports and retrospective studies have shown that both HHV-6 and HHV-7 are associated with a range of disease manifestations in transplant recipients. These include encephalitis, hepatitis and pneumonitis (Singh and Paterson, 2000; Ward *et al.*, 1989; Chan *et al.*, 1997). More importantly, these two viruses have also been associated with indirect effects including increasing the occurrence of HCMV disease and allograft rejection (Desjardin *et al.*, 1998; Osman *et al.*, 1996; Griffiths *et al.*, 1999a; Kidd *et al.*, 2000).

In prospective studies conducted at our centre in renal and liver transplant patients, qualitative and quantitative PCR were used to detect and measure HCMV, HHV-6 and HHV-7 loads in the blood (Griffiths *et al.*, 1999a; Kidd *et al.*, 2000). Clinicopathological analyses showed that both HHV-6 and HCMV were independently associated with biopsy proven rejection in the liver transplant patients (Griffiths *et al.*, 1999a), whereas in the renal transplant patients, HHV-7 was associated with increased episodes of graft rejection (Kidd *et al.*, 2000). One of the main aims of this thesis was to extend these prospective studies by utilising *in situ* techniques for the detection of these viruses directly in organ biopsies taken from the patients in the original cohorts to examine the pathogenic role of these viruses at the tissue level (see chapters 4 and 5).

The objectives of this chapter were therefore to develop specific, and more sensitive methods for *in situ* detection of human β-herpesvirus infected cells in tissue biopsies.

This chapter describes the construction of DIG-labelled riboprobes specific for the detection of HCMV gB, HCMV US28, HHV-6 U67, and HHV-7 U42 DNA in renal and liver biopsies. To further investigate HCMV gene expression in the renal biopsies a DIG labelled riboprobe directed against HCMV IE-1 gene transcripts was also developed. Preliminary experiments were also carried out to identify MAbs suitable for the detection of HCMV, HHV-6 and HHV-7 proteins by IHC on formalin-fixed paraffin-embedded culture infected cells. MAbs directed against HCMV proteins; HCMV IE protein, HCMV p52, and HCMV late protein, characteristic for each stage of HCMV replication were tested. The detection of HHV-6 specific proteins in HHV-6B infected cells were also tested using a range of MAbs directed to HHV-6A p41, HHV-6B p101, HHV-6B gH and HHV-6 gB. At present, only MAb 5E1 directed against HHV-7 pp85 (kindly provided by Dr Gabriella Campadelli-Fiume) has been found to maintain their reactivity in formalin-fixed paraffin-embedded tissues making it suitable for immunohistochemical detection of HHV-7.

3.2 Results

3.2.1 Immunohistochemistry (IHC)

3.2.1.1 IHC using the avidin-biotin complex (ABC) detection method on paraformaldehyde-fixed cell preparations

IHC using MAb directed to HCMV late protein, HHV-6B p101, and MAb 5E1 to HHV-7 pp85 were evaluated on paraformaldehyde-fixed HCMV infected MRC-5, HHV-6B infected Molt 3, and HHV-7 infected Sup T1 cells, respectively (section 2.2.1.1). The ABC detection method was developed by Hsu *et al.* (1981) and is based on the high affinity and essentially irreversible interaction between avidin and biotin. The technique requires pre-incubation of the biotinylated enzyme with the avidin, so that large complexes are formed for incubation with the biotinylated secondary Ab.

MAb to HCMV late protein and MAb 5E1 to HHV-7 pp85 at a 1:500 dilution showed strong positive staining of HCMV infected MRC-5, and HHV-7 infected Sup T1 cells respectively (figure 3.1). The DAB substrate produces a brown precipitate at the site of Ag detection. The intensity of staining increased with a 1:200 and 1:100 dilution of the MAbs, however, there was also an increase in background level. Therefore, optimal staining of these two MAbs was determined to be at a 1:500 dilution on paraformaldehyde-fixed cell preparations. A small number of positively stained HHV-6B infected cells were observed with MAb to HHV-6B p101 at a 1:200 dilution. A further increase to a 1:100 dilution increased the staining intensity and the number of positively stained HHV-6B infected cells detected. Therefore, optimal staining of MAb to HHV-6B p101 was determined to be at a 1:100 dilution on paraformaldehyde-fixed HHV-6 infected Molt-3 cells (figure 3.1).

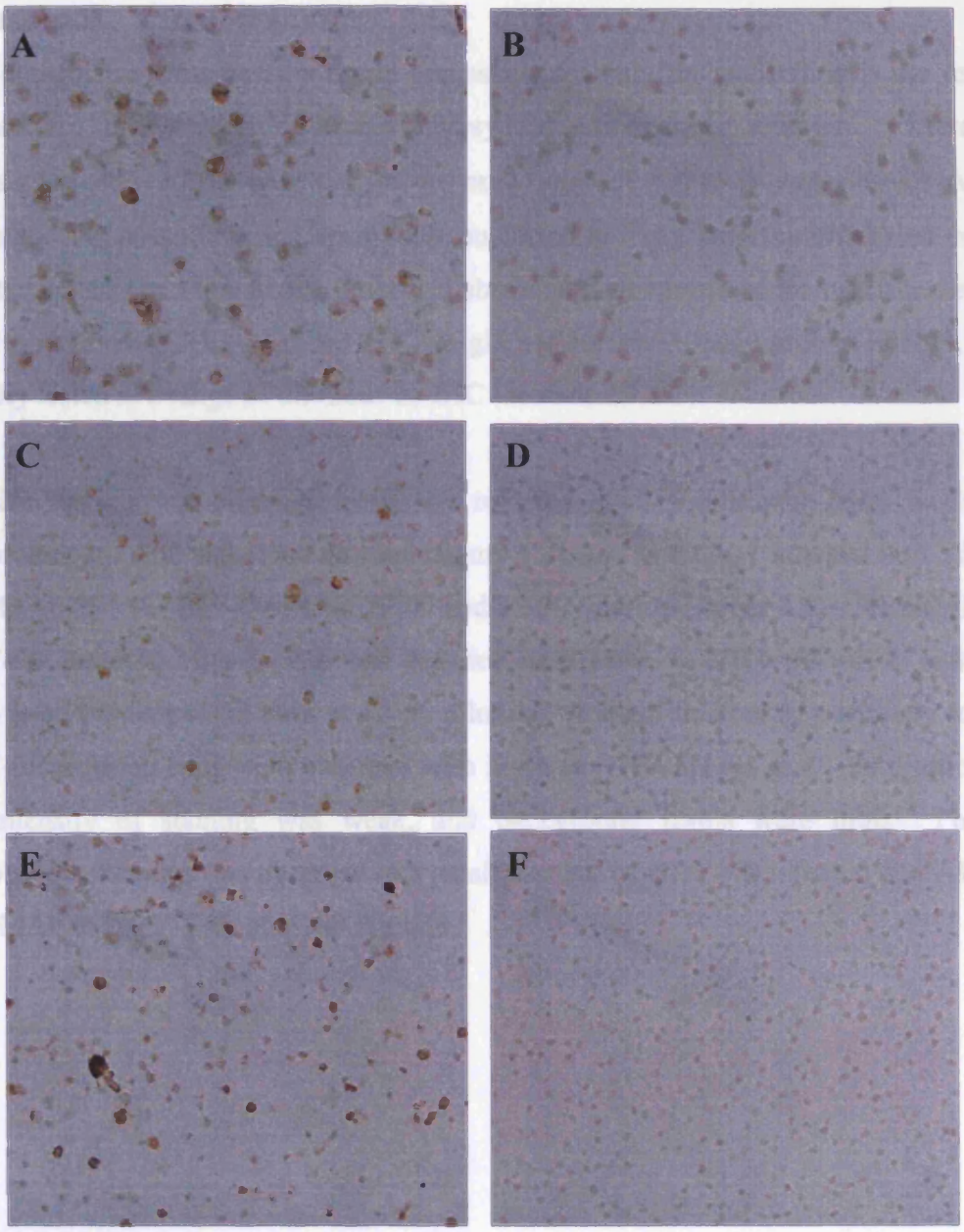


Figure 3.1 Immunohistochemical detection of the human β-herpesvirus proteins on paraformaldehyde-fixed cell preparations. A) Staining of HCMV infected MRC-5 cells and B) Negative control showing no staining in MRC-5 cells with MAB8126 to HCMV late protein (1:500 dilution); C) Staining of HHV-6B infected Molt-3 cells and D) Negative control showing no staining in Molt-3 cells with MAB8535 to HHV-6B p101 (1:100 dilution); E) Staining of HHV-7 infected Sup T1 cells and F) Negative control showing no staining in Sup T1 cells with MAb 5E1 to HHV-7 pp85 (1:500 dilution).

3.2.1.2 IHC using the ABC method on formalin-fixed paraffin-embedded culture infected cells

Formalin-fixation followed by tissue processing for paraffin embedding is the routine method for preservation of tissue biopsy for microscopic analysis. Therefore, preliminary experiments were carried out on a range of human β-herpesvirus MAbs to determine the sensitivity and specificity on formalin-fixed paraffin-embedded culture infected cells. The same MAbs described above, and an additional three MAbs directed to HHV-6 proteins; HHV-6 gB, HHV-6B gH and HHV-6A early protein p41/38, were initially tested at a range of dilutions by IHC (section 2.2.1.2).

Positive staining was observed in HCMV infected MRC-5 cells with MAb to HCMV late protein at 1:200 and 1:500 dilution (figure 3.2), and in HHV-7 infected Sup T1 cells with MAb 5E1 to HHV-7 pp85 at 1:200 and 1:500 dilution (figure 3.3). No staining of HHV-6B infected Molt-3 cells was detected with MAb to HHV-6B p101, and with MAb to HHV-6A p41/38 even at a 1:50 dilution. A small number of positively stained HHV-6B infected cells were observed with MAb to HHV-6B gH at a 1:50 dilution but the intensity of staining was weak, and background levels were high. Positive cytoplasmic staining was observed in a small number of HHV-6B infected Molt-3 cells with MAb to HHV-6 gB at 1:100 dilution.

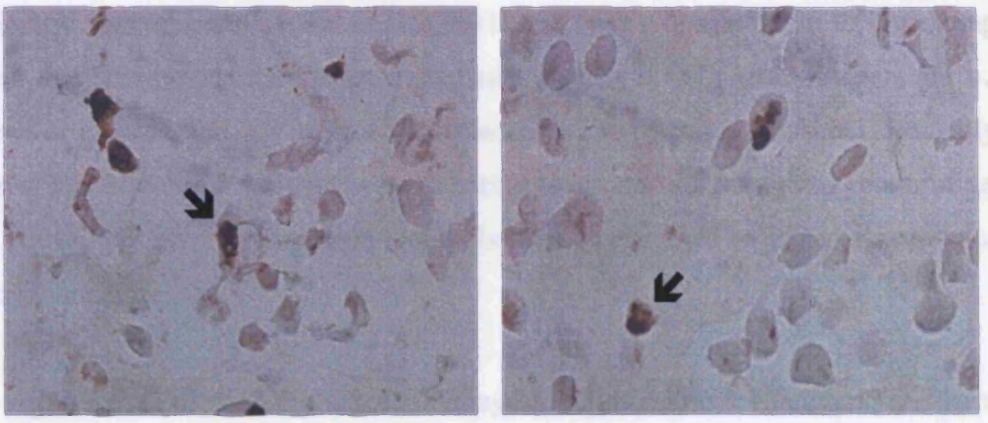


Figure 3.2 Immunohistochemical detection of HCMV late protein with MAB8126 (1:500 dilution) in formalin-fixed paraffin-embedded MRC-5 cells infected with HCMV (AD169).

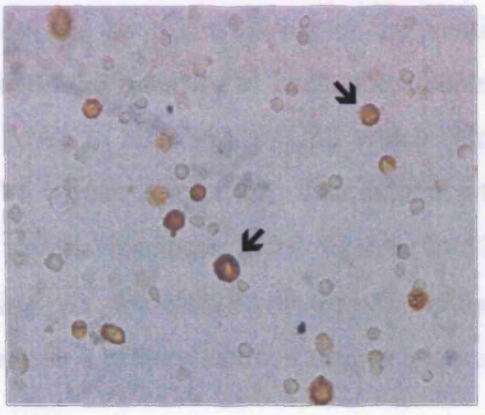


Figure 3.3 Immunohistochemical detection of HHV-7 pp85 with MAb 5E1 (1:500 dilution) in formalin-fixed paraffin-embedded Sup T1 cells infected with HHV-7.

3.2.1.3 Incorporation of an antigen retrieval method

The principle of Ag retrieval is to break the protein cross-links formed by formalin-or paraformaldehyde-fixation, and thereby unmasking hidden antigenic sites. This technology was reported by Shi *et al.* (1991) and uses a combination of high temp and buffers of different pH (the most commonly used is 0.1M Citrate buffer, pH 6.0) to retrieve or unmask the Ag in formalin-fixed paraffin-embedded tissues. Recently, Ag retrieval has also been applied to non-heating methods, including enzymatic digestion (Shi, 2001). Proteinase K treatment after fixation can be used to increase access to Ags, especially nuclear Ags.

Two Ag retrieval methods, proteolytic enzyme digestion, and heating in a microwave oven in Citrate buffer (as described in section 2.2.1.3), were incorporated into IHC. For each Ag retrieval method, MAbs directed to human β-herpesvirus late proteins (HCMV late protein, HHV-6 p101 and HHV-7 pp85), and an additional two MAbs directed to the HCMV IE protein and to HCMV p52 were tested at a range of dilutions, and staining intensities were compared on formalin-fixed paraffin-embedded culture infected cells.

Ag retrieval by microwaving or enzymatic digestion with Proteinase K significantly improved the intensity of immunohistochemical staining with the MAbs tested, except with MAb to HHV-6A p41/38 which remained negative as expected as this MAb is HHV-6 variant A specific. The number of virus-infected cells detected also increased. Five μg/ml Proteinase K for 30 mins at 37^0C was sufficient for immunohistochemical staining on formalin-fixed paraffin-embedded cells, and any further increase in proteinase K concentration did not significantly improve staining.

Optimal staining with MAbs to HCMV IE protein and to HCMV p52 were observed at 1:50 dilution with proteinase K digestion. Figure 3.4A shows detection of HCMV IE protein in HCMV infected MRC-5 cells. Figure 3.4B shows detection of HCMV p52 in the nucleus and cytoplasm of HCMV infected MRC-5 cells. Although the number of HCMV infected MRC-5 cells detected with the two MAbs at 1:200 dilution was increased with microwaving, background levels were higher and intensity of staining was less than that observed with proteinase K digestion. With MAb to HCMV late protein (1:200 dilution) and MAb 5E1 to HHV-7 pp85 (1:500 dilution) optimal staining

of respective HCMV infected MRC-5 and HHV-7 infected Sup T1 cells was observed with microwaving. Optimal staining was achieved at a 1:100 dilution (with Proteinase K digestion) and at a 1:200 dilution (with a microwaving step) with MAb to HHV-6 gB, and HHV-6B p101 (see figure 3.5 and 3.6).

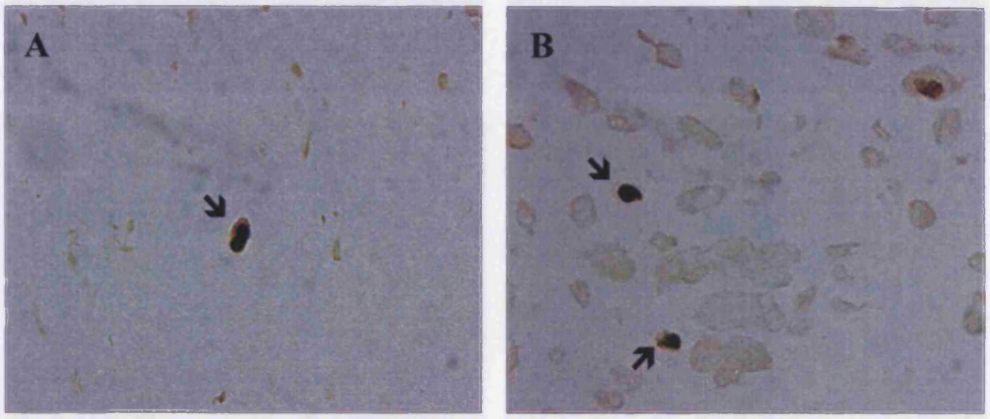
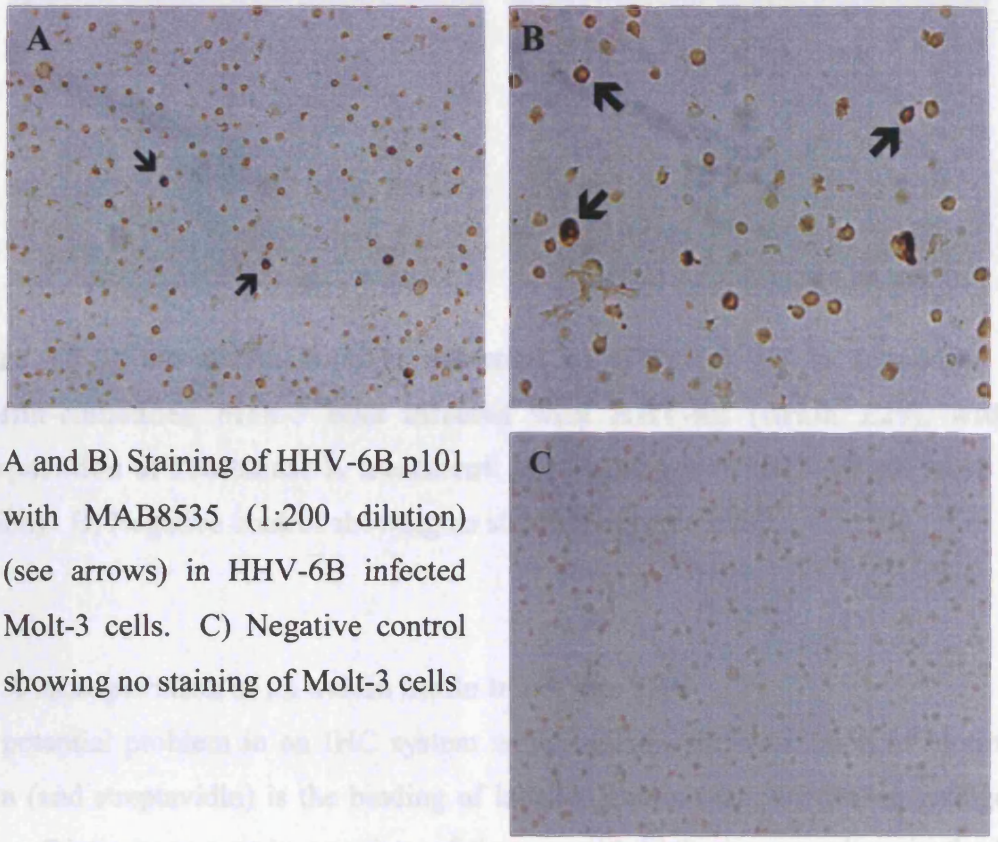


Figure 3.4 Immunohistochemical detection of HCMV proteins in formalin-fixed paraffin-embedded MRC-5 cells infected with HCMV (AD169). A) HCMV IE protein with MAB8131; and B) HCMV p52 with CCH2 MAb at 1 in 50 dilution, with Proteinase K digestion. Arrows indicate staining in MRC-5 cells infected with HCMV.



A and B) Staining of HHV-6B p101 with MAB8535 (1:200 dilution) (see arrows) in HHV-6B infected Molt-3 cells. C) Negative control showing no staining of Molt-3 cells

Figure 3.6 Immunohistochemical detection of HHV-6B p101 in formalin-fixed paraffin-embedded Molt-3 cells infected with HHV-6B (strain Z29), with the incorporation of a microwaving step.

Positive staining was observed with MAb to HHV-6B gH at 1:50 dilution with the incorporation of either Ag retrieval methods but background levels were high (see figure 3.7). MAb to HHV-6B gH was found not to be as sensitive and specific as that of MAb to HHV-6 gB and MAb to HHV-6B p101. Overall, incorporation of an Ag retrieval method significantly improved immunohistochemical staining of the human β-herpesvirus proteins. Generally, slightly higher background staining was observed with microwaving than with proteinase K digestion, and a higher concentration of MAb was required to achieve optimal staining when proteinase K was incorporated in IHC as compared to microwaving.

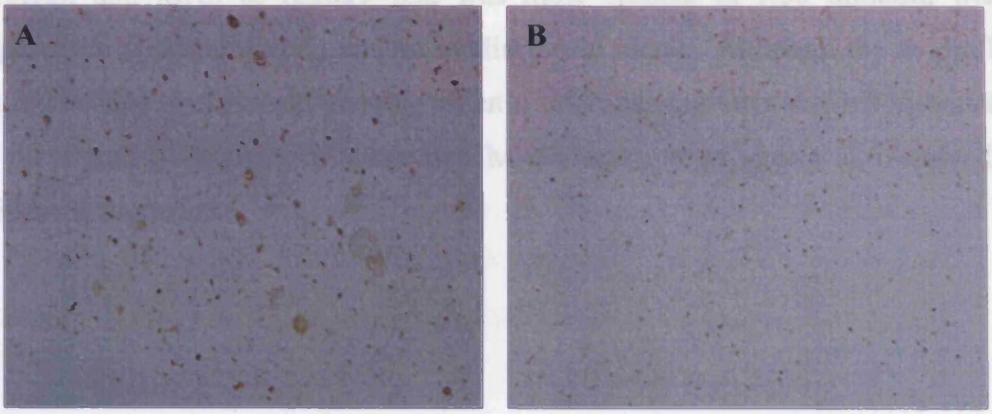


Figure 3.7 Immunohistochemical detection of HHV-6B gH in formalin- fixed paraffin-embedded Molt-3 cells infected with HHV-6B (strain Z29), with the incorporation of Proteinase K treatment. A) Staining with HHV-6B gH MAb (1:50 dilution). B) Negative control showing no staining of Molt-3 cells.

3.2.1.4 Incorporation of an avidin biotin block into IHC

One potential problem in an IHC system which utilizes the interaction of biotin with avidin (and streptavidin) is the binding of labelled avidin (streptavidin) to endogenous biotin. Biotin is present in a variety of tissues, with high concentrations in the brain, spleen, kidney, liver and lung tissues, hence excessive background staining and false-positive reactivity can occur. Therefore, to help reduce this background staining which was also most apparent when IHC was performed with a microwaving step, an avidin biotin blocking step was included as described in section 2.2.1.4.

IHC incorporating a microwaving step and an avidin biotin block was tested using a range of MAbs directed to the human β-herpesvirus proteins; HCMV IE protein, HCMV p52, HCMV late protein, HHV-6 gB, HHV-6B p101, and HHV-7 pp85 at a 1:100 and 1:200 dilution, on formalin-fixed paraffin-embedded cells infected with the respective virus. The incorporation of the avidin biotin blocking kit significantly reduced background levels for staining with MAb to HCMV late protein, MAb to HHV-6 gB, MAb to HHV-6B p101 and to MAb 5E1 to HHV-7 pp85 (see figure 3.8). All four MAbs gave good staining intensities at 1:200 dilution with low background. MAb to HHV-6 gB gave slightly better staining intensities and lower background levels as compared to MAb to HHV-6B p101. Immunohistochemical staining of MAb to HCMV

IE protein, and MAb to HCMV p52 was most optimal at 1:50 dilution, with the incorporation of proteinase K, and an avidin biotin block. Although the avidin biotin block did reduce background staining when a microwaving step was incorporated into IHC, the staining intensity of these two MAbs was not as strong as observed with proteinase K digestion.

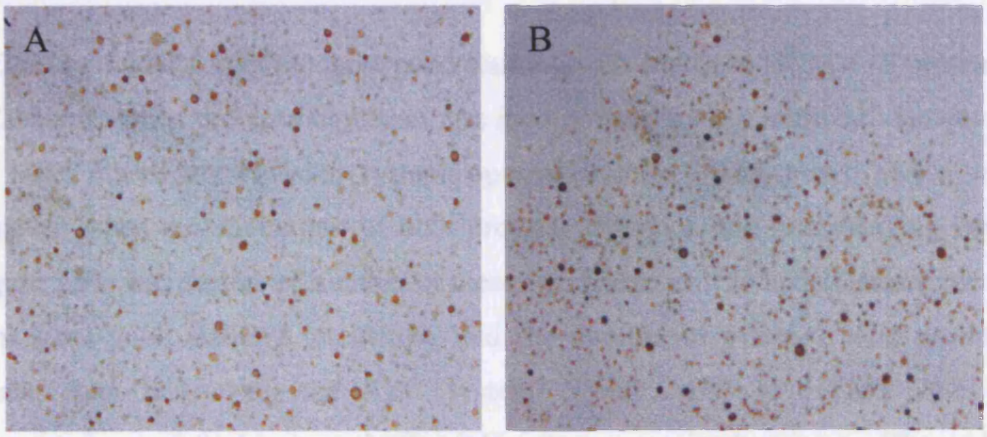


Figure 3.8 Immunohistochemical detection of HHV-7 pp85 in HHV-7 infected Sup T1 cells with MAb 5E1 at 1:200 dilution. A) Incorporation of an avidin biotin block. B) No avidin biotin block in IHC.

3.2.1.5 IHC using the Dako chemMate Envision detection system on formalin-fixed paraffin-embedded cells

The application of chain polymer technology (Dako chemMate Envision detection system) to improve the sensitivity of IHC detection was also tested. This system consists of a peroxidase-labelled dextran coupled with secondary Ab molecules directed against rabbit and mouse Igs. The same MAbs described in section 2.2.14 were tested on formalin-fixed paraffin-embedded culture infected cells (see section 2.2.2). This detection system has the advantage in that staining results are not affected by endogenous biotin.

IHC using the ABC method gave good staining with MAbs to HCMV IE protein and to HCMV p52 when protease digestion was incorporated for Ag retrieval. However, with the Dako chemMate envision system, optimal staining of these two MAbs was only achieved when a combination of both protease digestion and microwaving were used (figure 3.9). Incubation with either of these two MAbs at 1:50 and 1:100 dilution for 30 mins gave good staining intensities, and the number of infected cells detected was greater than that using the ABC system. With MAb to HCMV late protein, microwaving in 0.01 M citrate buffer (pH 6.0) gave optimal staining. The addition of protease treatment did not significantly improve staining. No positive staining was observed with 30 min incubation with MAb to HCMV late protein at 1:50 or 1:100 dilution.

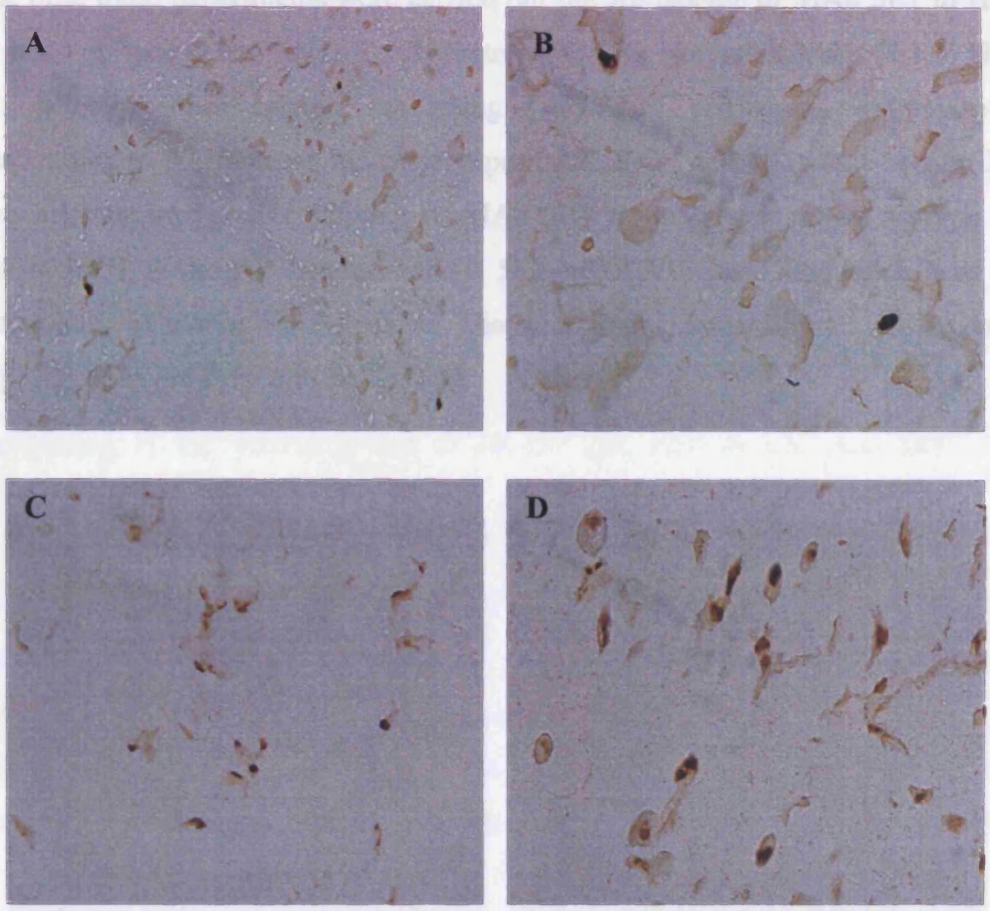


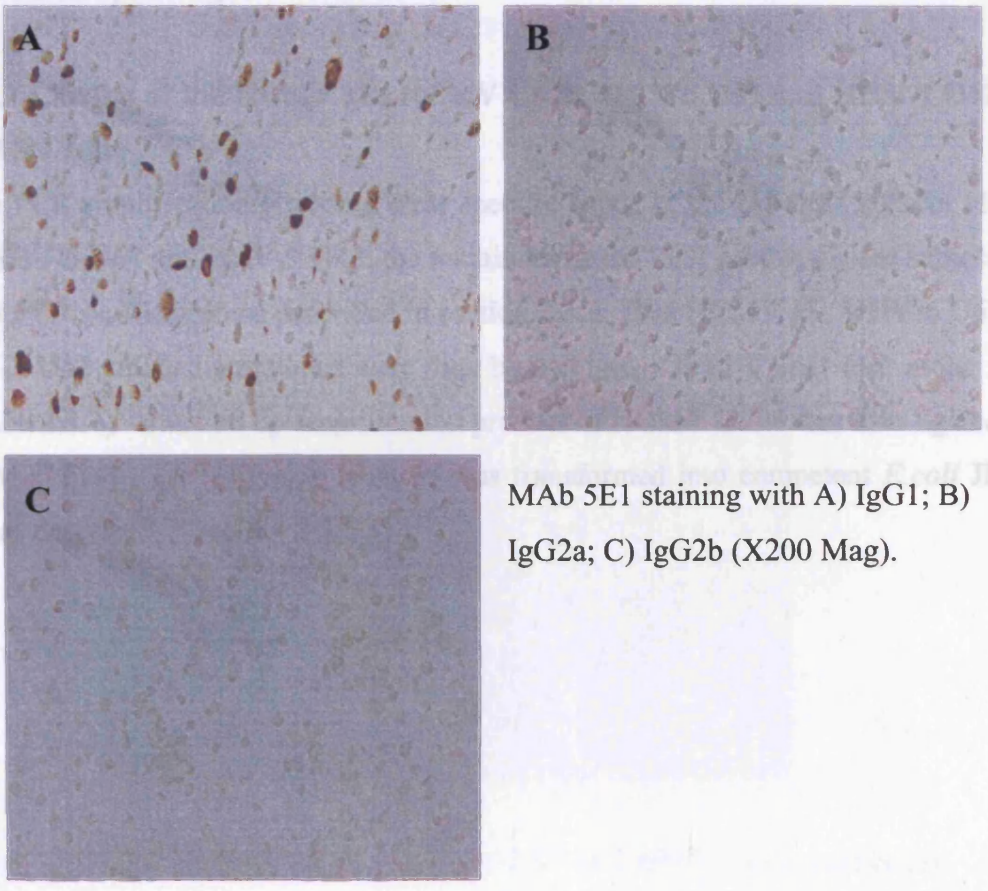
Figure 3.9 Immunohistochemical detection of HCMV proteins in formalin-fixed paraffin-embedded MRC-5 cells infected with HCMV (AD169) using the DAKO chemMate Envision detection kit. A and B) HCMV IE protein with MAB8131; and C and D) HCMV p52 with CCH2 MAb at 1 in 50 dilution.

3.2.1.6 Specificity of the MAbs

The specificity and crossreactivity of the immunohistochemical reaction with the MAbs described in section 2.2.1.4 were determined by testing each MAb against their respective virus and to the other human β-herpesvirus infected and non-infected cell controls. All MAbs tested reacted specifically with the respective virus, and did not display cross-reactivity to the heterologous virus. No positive staining was observed for non-infected cells (results not shown).

3.2.1.7 Subtyping of HHV-7 MAb 5E1

At the time these experiments were carried out the Ab isotype of MAb 5E1 to HHV-7 pp85 had not been determined. To characterize the Ab isotype of MAb 5E1 to HHV-7 pp85, detection was carried out using peroxidase conjugated antimouse Abs corresponding to the common mouse isotypes IgG_1, IgG_{2a} and IgG_{2b} (see section 2.2.4). Figure 3.10 shows positive staining of MAb 5E1 to pp85 with mouse isotype IgG1, therefore IgG1 is the Ab isotype. MAb 5E1 to HHV-7 pp85 has since been made commercially available by Advanced Biotechnologies International, USA, and the isotype has been confirmed to be IgG1.



MAb 5E1 staining with A) IgG1; B) IgG2a; C) IgG2b (X200 Mag).

Figure 3.10 Subtyping of HHV-7 mAb 5E1 on formalin-fixed paraffin-embedded HHV-7 infected Sup T1 cells using peroxidase conjugated anti-mouse Abs corresponding to common mouse isotypes.

3.2.2 *In situ* hybridisation (ISH) for detecting β-herpesvirus DNA

3.2.2.1 PCR amplification of HCMV gB, HHV-6 U67 and HHV-7 U42

A series of PCRs were set up to amplify HCMV gB, HHV-6 U67 and HHV-7 U42 control sequences as described in section 2.3.1. The HCMV gB, HHV-6 U67 and HHV-7 U42 control sequences differs from the wild-type HCMV gB, HHV-6 U67 and HHV-7 U42 target sequence by the presence of a restriction endonuclease site in the middle of the sequence (Fox *et al.*, 1992; Clark *et al.*, 1996; Kidd *et al.*, 1996). The PCR amplification products were separated by 3% agarose gel electrophoresis (figure 3.11 and 3.12) and amplification products of 149 bp, 173 bp and 143 bp were observed, corresponding to the predicted size of HCMV gB, HHV-6 U67 and HHV-7 U42 sequences, respectively.

3.2.2.2 Cloning of the HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 into pGEM-T Easy

Since PCR amplification produced clear specific bands of the expected size for HCMV gB, HHV-6 U67 and HHV-7 U42, the remainder of the PCR products were subjected to direct PCR purification as described in section 2.3.3. The HCMV gB, HHV-6 U67 and HHV-7 U42 purified amplicons were then ligated into pGEM-T Easy (1:1 molar ratio) (section 2.3.4). The 298 bp amplification product of HCMV US28 was also ligated into pGEM-T Easy. Each ligation reaction was transformed into competent *E.coli* JM109 cells as described in section 2.3.5.

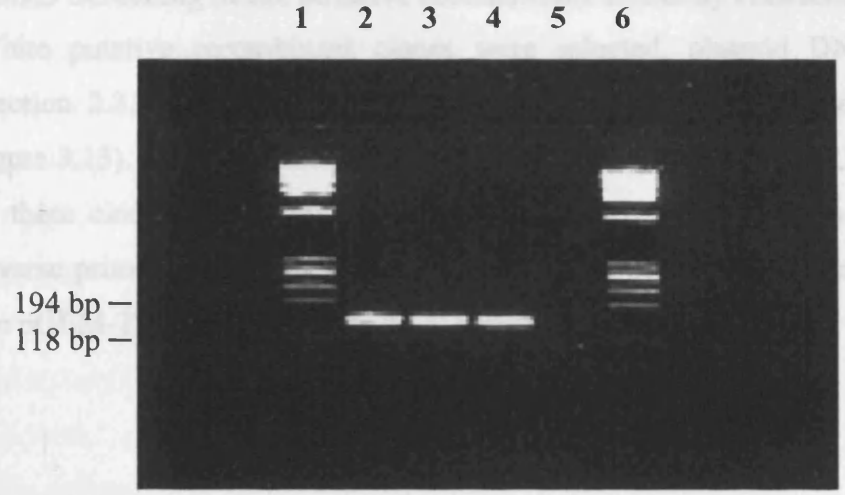


Figure 3.11 PCR amplification of HCMV gB sequence. Lanes 1 and 6) 0.5 µg PhiX174/*Hae*III Markers; 2-4) 10^8 copies of HCMV gB plasmid control; 5) Negative control-H_20

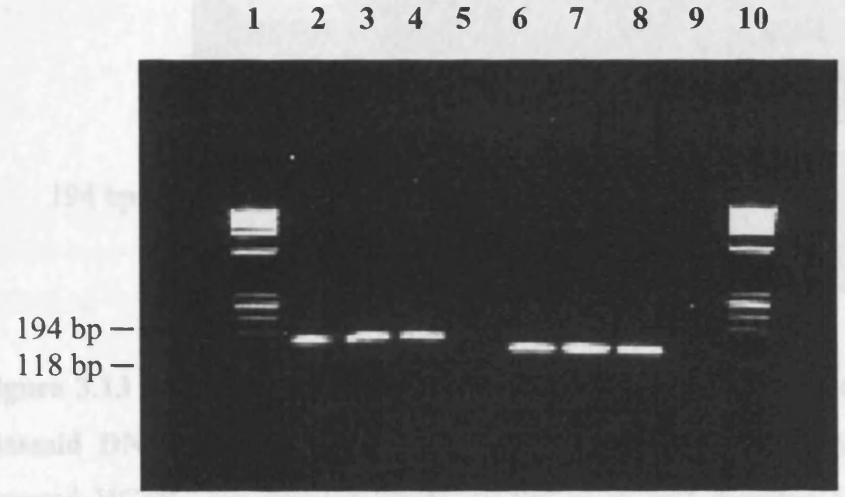


Figure 3.12 PCR amplification of HHV-6 U67 and HHV-7 U42 sequences. A) Lanes 1 and 10) 0.5 µg PhiX174/*Hae*III Markers; 2-4) 10^8 copies of HHV-6 U67 plasmid template; 6-8) 10^8 copies of HHV-7 U42 plasmid template; 5 and 9) Negative control-H_20.

3.2.2.3 Screening of the putative recombinant clones by restriction enzyme analysis

White putative recombinant clones were selected, plasmid DNAs were extracted (section 2.3.6), and analysed by restriction endonuclease digestion with *Eco*R1 (see figure 3.13). The presence of HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 in these clones was further confirmed by DNA sequencing with M13 universal and reverse primers. From the sequencing results, all inserts were found to be cloned into the pGEM-T Easy vector in the 5'-3' orientation.

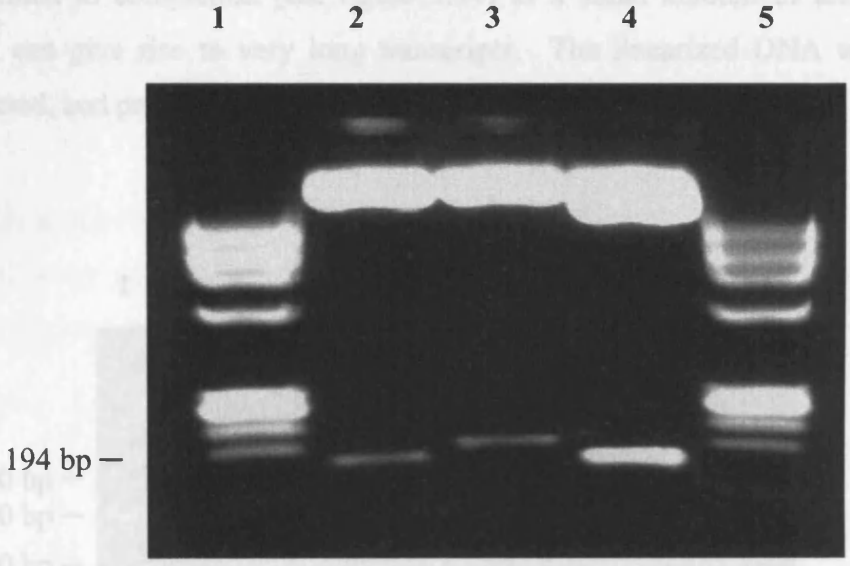


Figure 3.13 *Eco*RI restriction digests of HCMV gB, HHV-6 U67 and HHV-7 U42 plasmid DNA. Lanes 1 and 5) 0.5 µg PhiX174/*Ha* III Markers; 2) 300 ng *Eco*RI digested HCMV gB plasmid DNA; 3) 300 ng *Eco*RI digested HHV-6 U67 plasmid DNA; and 4) 300 ng *Eco*RI digested HHV-7 U42 plasmid DNA

3.2.2.4 Linearization of plasmid DNAs with restriction enzyme for the production of "run-off" transcripts

Once the HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 inserts had been successfully cloned into pGEM-T Easy, the recombinant plasmids were linearized with restriction enzymes to generate "run-off" transcripts. The plasmid DNAs were linearized with restriction enzymes, *Nde*I or *Nco*I (section 2.3.8). These two restriction enzymes were chosen as they have only one restriction site in the pGEM-T Easy vector, and no restriction site in the inserts. In addition, they do not leave a 3' overhang on the template, which can result in extraneous transcripts that contain sequences complementary to the expected transcript as well as sequences corresponding to vector DNA. A 0.8% agarose gel was used to confirm that the restriction digest had been performed to completion (see figure 3.14) as a small amount of undigested plasmid DNA can give rise to very long transcripts. The linearized DNA was subsequently extracted, and precipitated (section 2.3.9) for *in vitro* transcription.

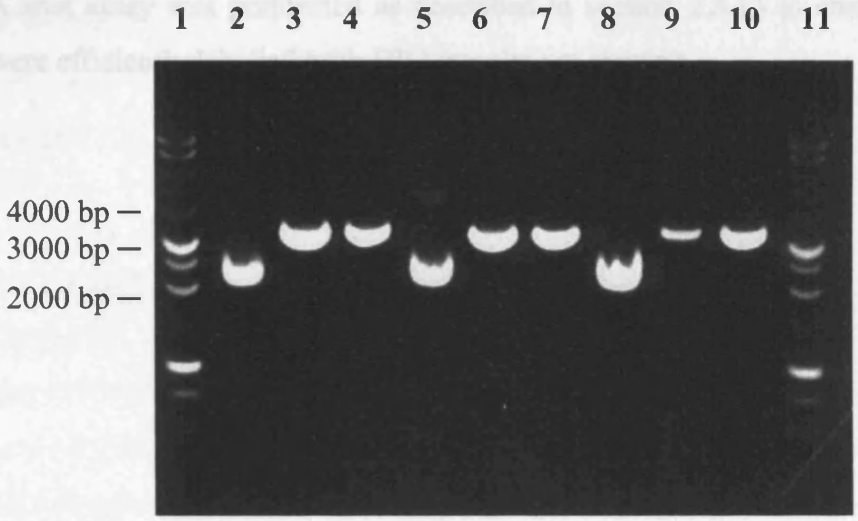


Figure 3.14 Linearization of HCMV gB, HHV-6 U67 and HHV-7 U42 plasmid DNA with *Nco*I or *Nde*I. Lanes 1 and 11) 0.5 µg 1 kb DNA ladder; 2, 5 and 8) 300 ng HCMV gB, HHV-6 U67 and HHV-7 U42 plasmid DNA; 3, 6 and 9) 300 ng *Nco*I digested HCMV gB, HHV-6 U67 and HHV-7 U42 plasmid DNA; 4) 300 ng *Nde*I digested HCMV gB, HHV-6 U67 U42 and HHV-7 U42 plasmid DNA.

3.2.2.5 Generation of HCMV, HHV-6 and HHV-7 RNA transcripts

Since HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 were cloned into pGEM-T Easy in the 5'-3' orientation, *in vitro* transcription was performed using T7 and Sp6 RNA polymerase on *Nde*I and *Nco*I linearized DNAs, to generate sense and antisense transcripts, respectively (2.3.10). The RNA transcripts were subsequently ethanol precipitated (section 2.3.11) and analysed on a 3% denaturing agarose gel. Figure 3.15 shows *in vitro* transcription of; HCMV gB template DNA with Sp6 and T7 RNA polymerase giving rise to the expected band sizes of 255 bp and 250 bp respectively; HHV-6 U67 template with Sp6 and T7 RNA polymerase giving rise to the expected band sizes of 279 bp and 274 bp respectively; HHV-7 U42 template with Sp6 and T7 RNA polymerase giving rise to the expected band sizes of 249 bp and 244 bp respectively. *In vitro* transcription of HCMV US28 template DNA with Sp6 and T7 RNA polymerase gave rise to the expected band sizes of 404 bp and 399 bp, respectively (results not shown).

3.2.2.6 Spot assay to confirm DIG labelling of RNA transcripts

A spot assay was performed as described in section 2.3.13 to ensure RNA transcripts were efficiently labelled with DIG (results not shown).

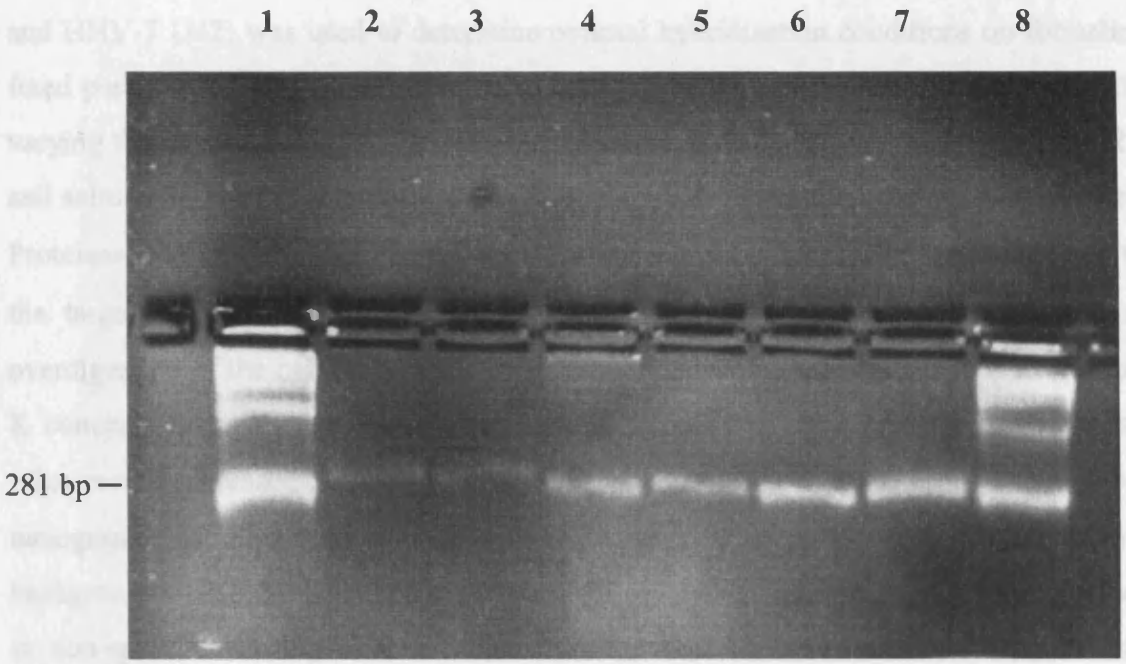


Figure 3.15 *In vitro* transcription of HCMV gB, HHV-6 U67 and HHV-7 U42 template DNA with Sp6 and T7 RNA polymerase to generate antisense and sense RNA transcripts. Lanes 1 and 8) 1 µg RNA Markers; 2-7) 5 µl of HCMV gB, HHV-6 U67 and HHV-7 U42 transcription reaction with T7 and Sp6 RNA polymerase respectively.

3.2.2.7 ISH on 4% paraformaldehyde-fixed cell preparations

Using ISH, DIG labelled riboprobes specific for HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 were evaluated for detecting the presence of HCMV, HHV-6 and HHV-7 DNA respectively, in paraformaldehyde-fixed cell controls (see section 2.4.1). HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 DNA were localised in the nuclei of HCMV infected HEL, HHV-6 infected Molt 3, and HHV-7 infected SupT1 cells respectively (results not shown). Hybridisation signals were observed as dark purple spots within the nucleus of infected cells. Pre-treatment of paraformaldehyde-fixed cell preparations with no proteinase K or low concentrations of Proteinase K (2.5-10 µg/ml) for 30 mins at 37^0C was sufficient for probe penetration to the target. Too high a concentration of Proteinase K (50-200 µg/ml) resulted in over-digestion of the cells and lower hybridisation signals.

3.2.2.8 ISH on formalin-fixed paraffin-embedded culture infected cells

ISH with each of the DIG labelled riboprobes (HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42) was used to determine optimal hybridisation conditions on formalin-fixed paraffin-embedded culture infected cells. This was determined by independently varying the concentration of: proteinase K (section 2.4.2.1); riboprobe (section 2.4.2.2); and salmon sperm DNA and yeast tRNA (section 2.4.2.3) in the hybridisation mixture. Proteinase K (2.5-5 μg/ml) at 37^0C for 30 mins was sufficient for probe penetration to the target. An increase in Proteinase K concentration to 100 μg/ml resulted in overdigestion of the cells and lower hybridisation signals. Once the optimal Proteinase K concentration was determined, the concentration of riboprobe in the hybridisation mixture was increased from 5 to 10 ng riboprobe per 50 μl of hybridisation mix. Five nanograms of riboprobe gave good hybridisation signals with relatively low backgrounds. Riboprobe concentrations of 7.5 ng and 10 ng were too high and resulted in non-specific staining of cells. Once the optimal concentration of riboprobe was determined, the concentration of salmon sperm DNA and yeast tRNA was increased to 1 mg/ml per hybridisation reaction. One mg/ml of each nucleic acid per hybridisation reaction gave good hybridisation signals and the level of non-specific background was reduced. The exclusion of nucleic acids in the hybridisation mixture resulted in much higher background levels (results not shown). Figure 3.16 shows localisation of HCMV, HHV-6 and HHV-7 DNA in the nucleus of formalin-fixed paraffin-embedded cells infected with the respective virus. Formalin-fixed paraffin-embedded cell controls were treated with 2.5 μg/ml Proteinase K at 37^0C for 30 min. Hybridisation was performed at 37^0C with 5 ng riboprobe and 1 mg/ml salmon sperm DNA and yeast tRNA incorporated per hybridisation reaction.

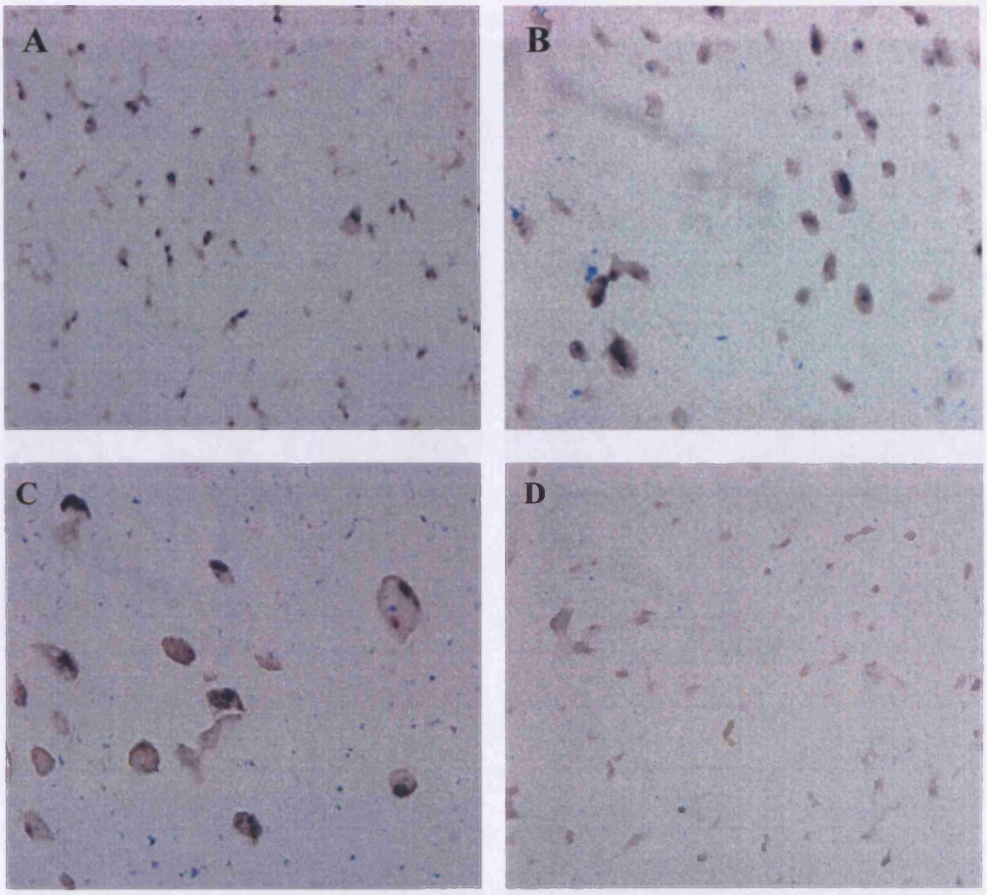
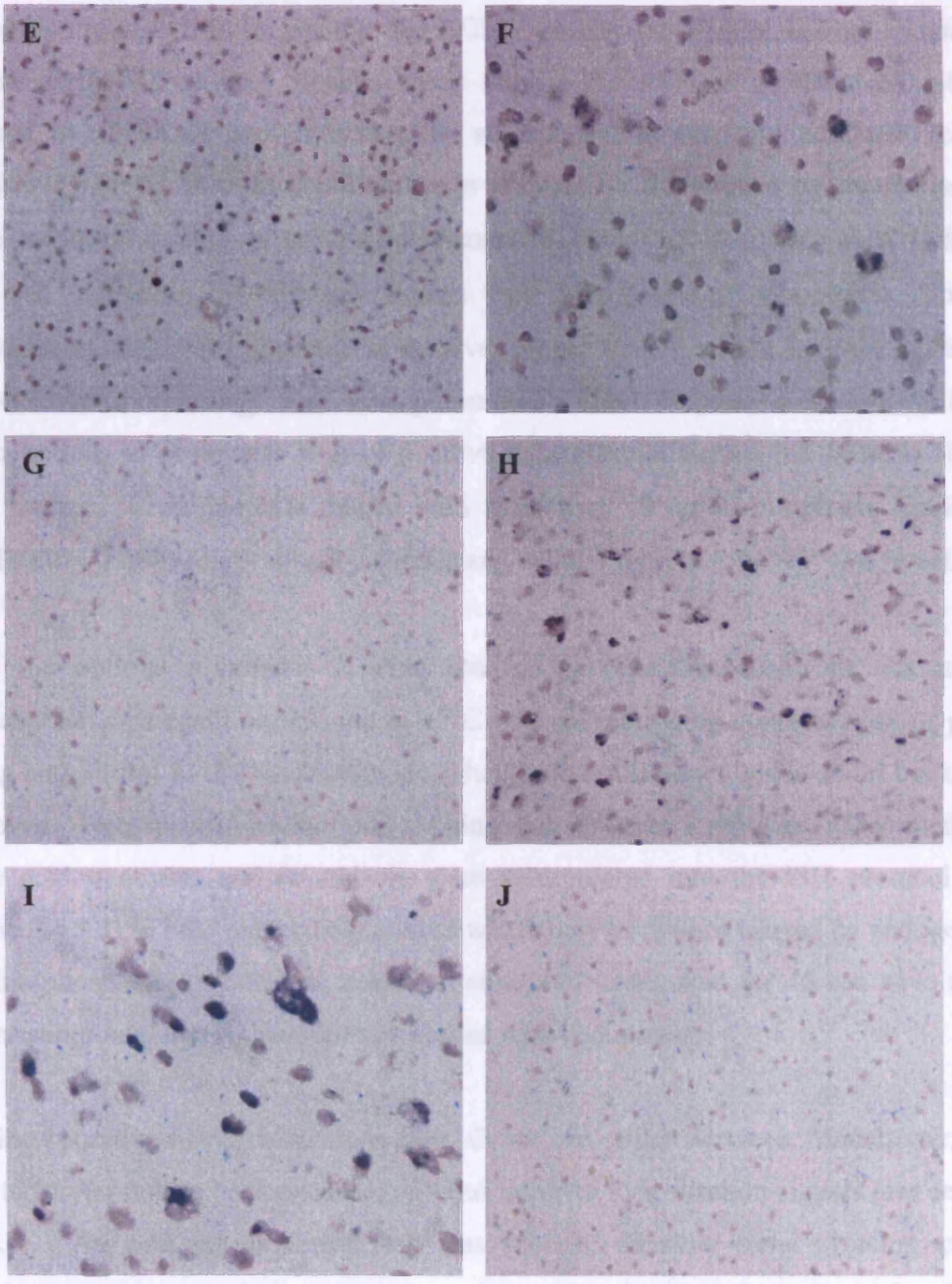


Figure 3.16 Detection of human β-herpesvirus DNA in formalin-fixed paraffin-embedded culture infected cells by ISH. A positive reaction is indicated by dark purple spots within the nuclei of infected cells. A) and B) HCMV gB DNA in HCMV infected HEL cells; C) shows HCMV US28 DNA in HCMV infected HEL cells; D) Negative control showing no staining of HEL cells. E) and F) HHV-6 U67 DNA in HHV-6B infected cells; G) Negative control showing no staining in Molt-3 cells. H and I) HHV-7 U42 in HHV-7 infected Sup T1 cells; I) Negative control showing no staining in Sup T1 cells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3.2.2.9 Optimisation of ISH on formalin-fixed paraffin-embedded liver biopsies

To optimise ISH conditions for the detection of the human β-herpesviruses in liver biopsies, a biopsy which tested positive for HCMV late protein 47-55 kDa by IHC (and independently shown to be positive for HCMV p52 by the Histopathology Diagnostic Service at the Royal Free Hospital) was used. The level of proteolytic treatment required to obtain optimal hybridisation signals and to maintain adequate cellular morphology in the liver biopsy tissue was empirically determined by incubating the tissue section at a range of proteinase K concentrations (2.5-15 μg/ml) at 37^0C for 30 min, and comparing hybridisation signals with the HCMV gB riboprobe. Optimal hybridisation signal was observed in the liver biopsy treated with 7.5 μg/ml proteinase K. Cellular morphology was also preserved. Liver biopsies treated with lower concentrations of proteinase K gave positive hybridisation signals but positive signals were weaker. Liver biopsies treated with more than 10 μg/ml proteinase K did not significantly improve hybridisation signals and cellular morphology was less intact.

Once the optimal proteinase K concentration was determined to be 7.5 μg/ml, hybridisation was again carried out at 37^0C with an increasing concentration of probe (10 ng and 20 ng) in the reaction to see whether hybridisation signals could be further improved. Non-specific background staining was however a problem. Two methods, acetic acid treatment and levamisole were incorporated into the ISH protocol (see section 2.4.3.3) to help reduce background which may have been caused by endogenous alkaline phosphatase. Sections treated in cold 20% acetic acid for 45 sec gave much lower background levels than those incubated with levamisole.

ISH incorporating recommendations from Orion Molecular Services, Manchester, was used to further reduce background levels and improve hybridisation signals (see section 2.4.3.4). An acid pre-treatment step was used to denature basic proteins and in combination with proteolytic treatment can be used to facilitate their removal and thus making the target more accessible. After proteinase K treatment, sections were post-fixed in 0.4% paraformaldehyde in PBS for 20 min to help prevent further loss of target sequences and disintegration of the tissue. Hybridisation with increasing riboprobe concentration was carried out at 42^0C overnight to help improve the hybridisation signal. Post hybridisation washes were also more stringent to increase specificity of the probe hybridisation and to reduce background associated with non-specific

hybridisation. ISH using these recommendations significantly reduced background levels previously observed in the liver biopsies. Hybridisation signals were also further improved when hybridisation was carried out at 42^0C overnight with 50 ng HCMV gB riboprobe. Using this optimised method, liver biopsies from transplant patients were tested for human β-herpesvirus DNA, and the results are presented in chapter 5.

3.2.2.10 Optimisation of ISH on formalin-fixed paraffin-embedded renal biopsies

Using the same optimised ISH conditions as for the liver biopsies, 10 formalin-fixed paraffin-embedded renal biopsies taken from transplant patients suspected of graft dysfunction were initially tested for HCMV gB DNA. Of the 10 renal biopsies tested, 5 were positive for HCMV gB DNA. One of these HCMV gB DNA positive samples was then used to determine the proteinase K concentration required for efficient probe penetration to target nucleic acids in renal biopsy tissue. The renal biopsy was pretreated with a range of concentrations of Proteinase K (2.5-15 μg/ml) and hybridisation signals were compared. As with liver biopsy tissue, renal biopsy treated with 7.5 μg/ml proteinase K for 30 min at 37^0C gave optimal hybridisation signals together with good preservation of cellular morphology (results not shown).

3.2.2.11 Specificity of the HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 riboprobes

HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 DIG labelled riboprobes were tested against formalin-fixed paraffin-embedded cells infected with respective virus and to each of the other human β-herpesviruses to test for cross-reactivity. None of the riboprobes reacted with the heterologous viruses, and non-infected cell controls stained negative (results not shown).

3.2.2.12 Construction of a human β-globin riboprobe to determine the sensitivity of ISH

Using PCR a human β-globin sequence was amplified (see section 2.4.6.1) and the resulting amplicon was separated by 3% agarose gel electrophoresis. Figure 3.17 shows an amplification product of 110 bp corresponding to the predicted size of the human β-globin sequence. The remainder of the PCR product was subjected to direct PCR purification, and cloned into the pGEM-T Easy vector (section 2.4.6.1-2.4.6.2).

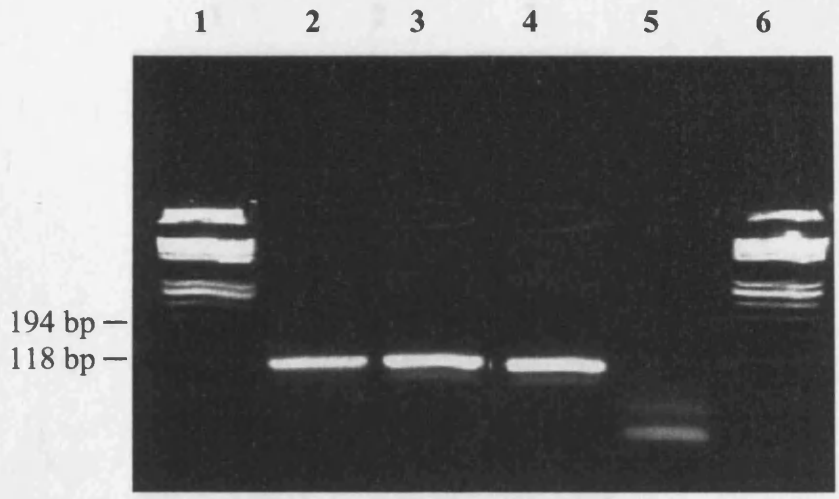


Figure 3.17 PCR amplification of a 110 bp human β-globin sequence Lanes 1 and 6) 0.5 µg PhiX174/*Hae*III Markers; 2-4) 100 ng of human DNA; 5) Negative control-H_20

A white putative recombinant clone was selected, plasmid DNA was extracted and analysed by restriction digestion with *Eco*RI to show that the 110 bp human β-globin sequence had been cloned into pGEM-T Easy (figure 3.18). The plasmid DNA was also sequenced with M13 universal and reverse primers to confirm the correct insert. The sequencing results showed that the insert was cloned into the pGEM-T Easy vector in the 3'-5' orientation. Therefore, the plasmid DNA was linearized with restriction enzymes *Nde*I and *Sph*I (see figure 3.19) for subsequent *in vitro* transcription with T7 and Sp6 RNA polymerases, respectively to generate antisense and sense RNA transcripts for ISH. Figure 3.20 shows *in vitro* transcription of the human β-globin template DNA with T7 and Sp6 RNA polymerases to give rise to the expected band sizes of 211 bp and 221 bp, respectively. A spot assay was performed as described in section 2.3.13 to ensure RNA transcripts were labelled with DIG.

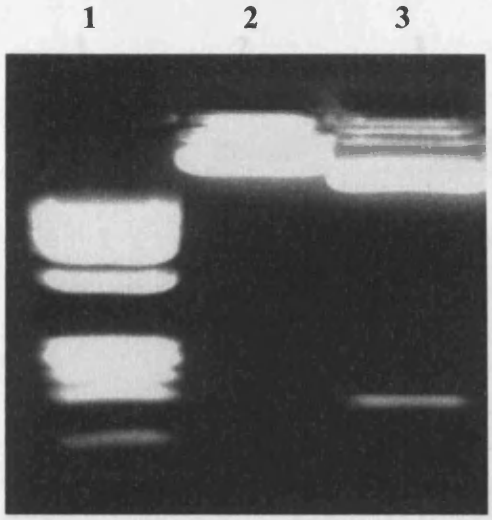


Figure 3.18 *Eco*RI restriction digest of human β-globin plasmid DNA. Lane 1) 0.5 μg PhiX174/*Hae*III Marker; 2) 300 ng human β-globin plasmid DNA; 3) 300 ng *Eco*RI digested human β-globin plasmid DNA.

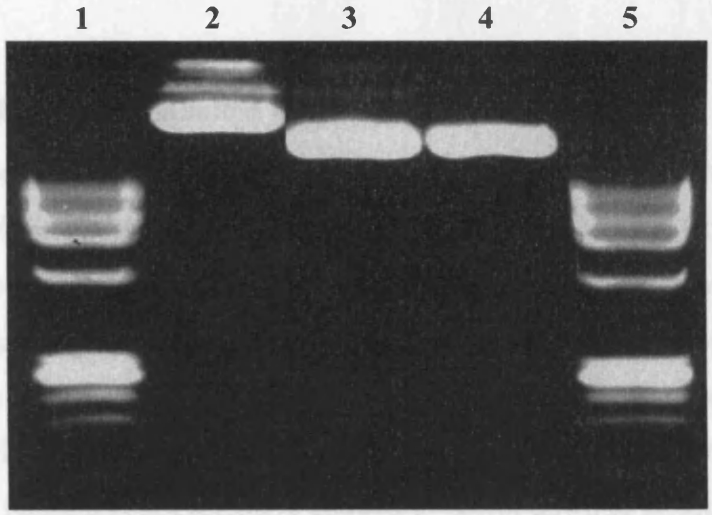


Figure 3.19 Linearization of human β-globin plasmid DNA with *Nde*I and *Sph*I. Lanes 1 and 5) 0.5 μg PhiX174/*Hae*III Marker; 2) 300 ng human β-globin plasmid DNA; 3) and 4) 300 ng *Nde*I and *Sph*I digested human β-globin plasmid DNA

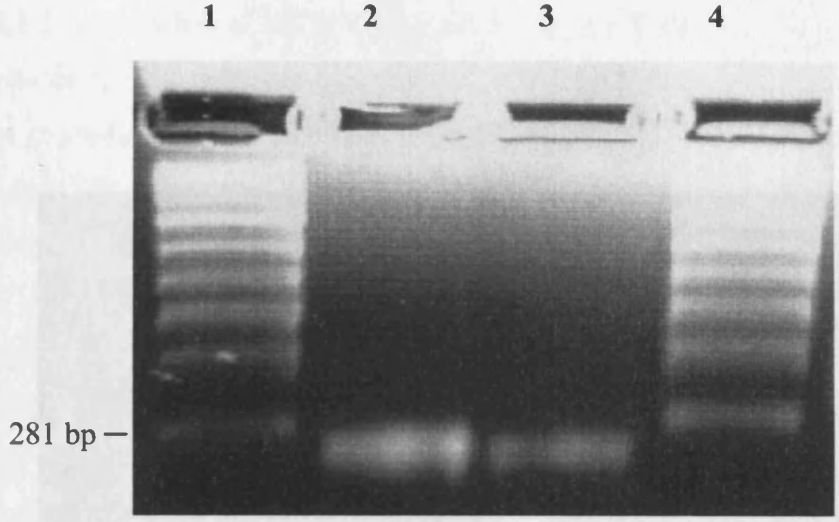


Figure 3.20 *In vitro* transcription of human β-globin template DNA with Sp6 and T7 RNA polymerase to generate sense and antisense RNA transcripts. Lanes 1 and 5) 1 μg RNA Markers; 2 and 3) 5 μl of β-globin transcription reaction with T7 and Sp6 RNA polymerase, respectively.

3.2.2.13 ISH with DIG labelled human β-globin riboprobe

A human β-globin riboprobe was constructed as described in section 2.4.6.3 to test the sensitivity of ISH. Human β-globin DNA is present as 2 copies per cell, therefore using the human β-globin sense probe which will only detect DNA (and no mRNA), the level of sensitivity of hybridisation can be determined. No positive hybridisation signal was observed on 4% paraformaldehyde fixed HEL cells or on formalin-fixed paraffin-embedded HEL cells with this probe using optimised ISH conditions. This suggests that the ISH method is not sensitive enough to detect down to single copy levels.

3.2.2.14 Determining the level of sensitivity of HHV-6 ISH using a lymphoblastoid cell line with integrated HHV-6 genome (V1-LCL)

The sensitivity of ISH using HHV-6 U67 riboprobe was tested on a lymphoblastoid cell line with integrated HHV-6 genome (V1-LCL). Depending on the QC-PCR used, V1-LCL was found to contain 12 or 26 genome copies per cell (results not shown). Positive staining of HHV-6 infected cells were detected by ISH. However, not all cells stained positive (see figure 3.21).

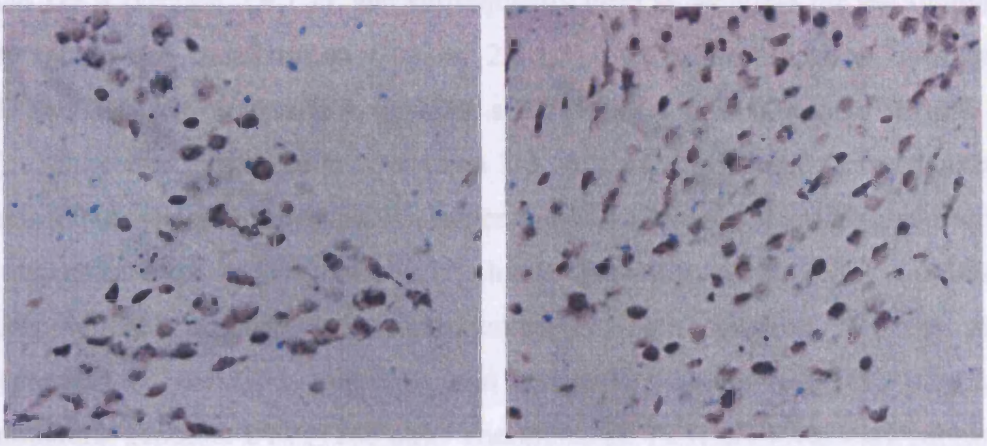


Figure 3.21 HHV-6 ISH on formalin fixed paraffin embedded V1-LCL. Positive nuclear staining observed in >50% of cells.

3.2.3 *In situ* hybridisation (ISH) for detecting HCMV gene expression

3.2.3.1 Generation of HCMV IE-1 cDNA by RT-PCR

Cultures of HEL cells infected with HCMV were harvested 4 hrs after infection. RNA was extracted (section 2.6.1.1), treated with RQ1 RNase-Free DNase to remove any residual DNA (section 2.6.1.2), and subsequently used as template for the synthesis of cDNA by reverse transcription (section 2.6.1.3). PCR amplification with primers IEP3D and IEP2AII were used to generate a HCMV IE-1 gene transcript (Kondo *et al.*, 1994). These two primer pairs span exon 2-3, so that amplification of contaminating genomic DNA yields a substantially larger product (263 bp) than the expected IE-1 gene transcript (151 bp). Figure 3.22 shows separation of the PCR amplification products by 3% agarose gel electrophoresis. The expected 151 bp product corresponding to the IE-1 transcript was observed together with the presence of contaminating genomic DNA (263 bp). Therefore, the PCR products were subjected to low-gelling point agarose gel electrophoresis, and the band corresponding to the size of HCMV IE-1 (151 bp) was excised from the gel and purified (section 2.6.1.5).

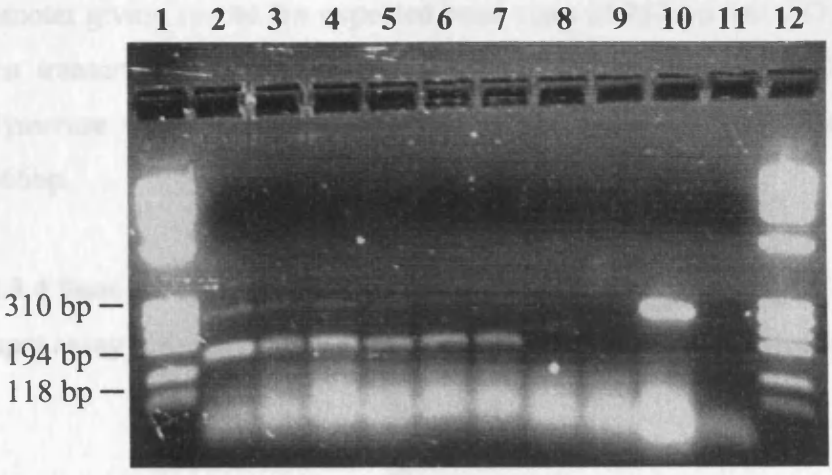


Figure 3.22 PCR amplification with primers HCMV IEP3D and IEP2AII. Lanes 1 and 12) 0.5 µg PhiX174/*Hae*III Markers; 2-7) 0.5 µg HCMV cDNA; 8) 0.5 µg uninfected HEL cDNA; 9) 0.5 µg HCMV RNA (no RT step); 10) 0.5 µg HCMV RNA with no DNase treatment and no RT step, and 11) H_20.

3.2.3.2 Cloning of the HCMV IE-1 gene into pGEM-T Easy

The HCMV IE-1 purified amplicon was then ligated into pGEM-T Easy (1:1 molar ratio) as described in sections 2.3.4. The ligation reaction was transformed into competent *E.coli* JM109 cells (section 2.3.5). A white putative recombinant clone was selected and purified as described in section 2.5.7.

3.2.3.3 Restriction enzyme analysis of the putative recombinant clone

The plasmid DNA was extracted and analysed by restriction enzyme digestion with *Eco*RI as described in section 2.3.7. The digest showed that the clone selected contained the HCMV IE-1 gene insert (figure 3.23). This was further confirmed by DNA sequencing with M13 universal primers. The results showed that the HCMV IE-1 gene was cloned into the pGEM-T Easy vector in the 3'-5' orientation. The plasmid DNA was then linearized with *Nde*I and *Nco*I restriction enzyme (see figure 3.24), and *in vitro* transcribed with T7 and Sp6 RNA polymerase to generate antisense and sense RNA transcripts respectively. The RNA transcripts were subsequently ethanol precipitated and analysed on a 3% denaturing agarose gel. Figure 3.25 shows *in vitro* transcription of HCMV IE-1 template DNA with Sp6 and T7 RNA polymerase promoter giving rise to the expected band sizes of 257 bp and 252 bp, respectively. *In vitro* transcription of the pGEM Express Positive control template with Sp6 RNA polymerase was included giving rise to the expected band sizes of 1,787 bp and 2,566bp.

3.2.3.4 Spot assay to confirm DIG labelling of RNA transcripts

A spot assay showed the RNA transcripts were efficiently labelled with DIG.

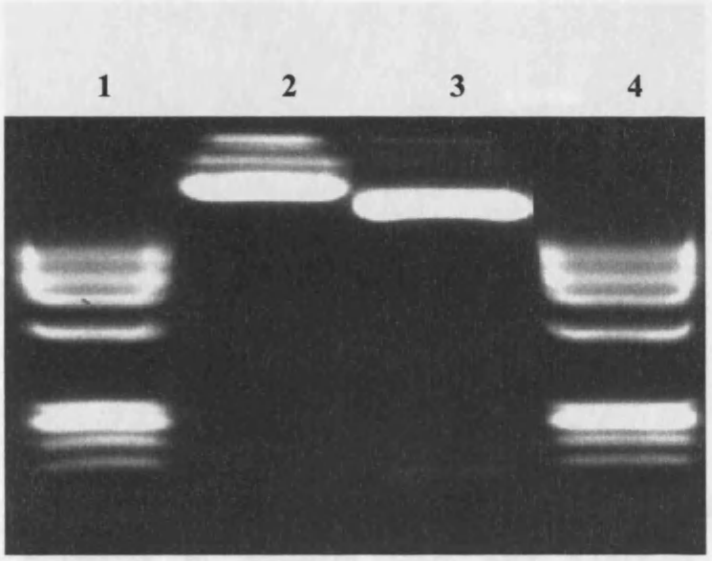


Figure 3.23 *Eco*RI restriction digest of HCMV IE-1 plasmid DNA Lanes 1 and 4) 0.5 µg PhiX174/*Hae*III Markers; 2) 300 ng HCMV IE-1 plasmid DNA; 3) 300 ng *Eco*RI digested HCMV IE-1 plasmid DNA

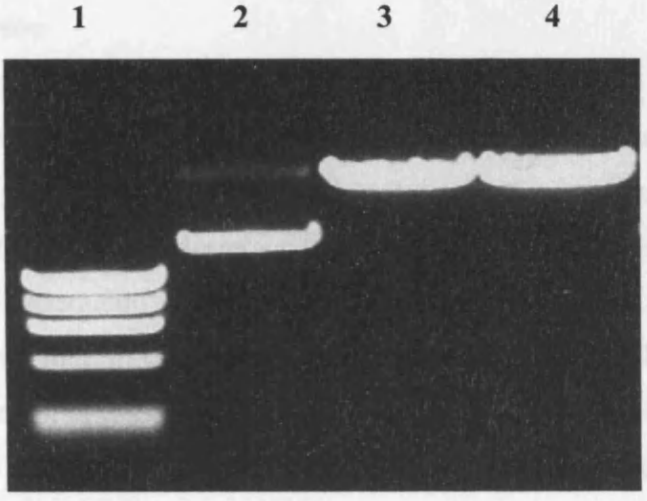


Figure 3.24 Restriction digests of HCMV IE-1 plasmid DNA. Lane 1) 0.5 µg PhiX174/*Hae*III Marker; 2) 300 ng HCMV IE-1 plasmid; 3) 300 ng *Nco*I digested HCMV IE1 plasmid DNA; 4) 300 ng *Nde*I digested HCMV IE-1 plasmid DNA

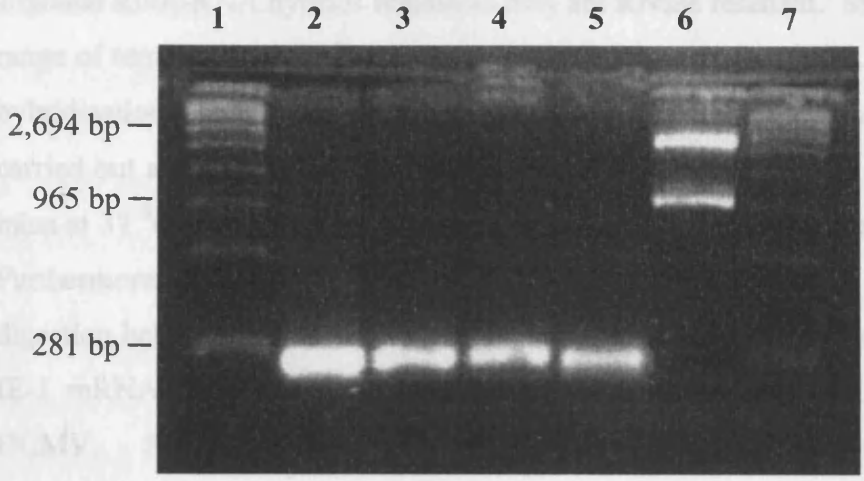


Figure 3.25 *In vitro* transcription of HCMV IE-1 template with Sp6 and T7 RNA polymerase promoter to generate sense and antisense RNA transcripts, respectively. Lanes 1 and 7) 1 µg RNA Markers; 2 and 3) 5 µl of HCMV IE-1 transcription reaction with Sp6 RNA polymerase; 4 and 5) 5 µl of HCMV IE-1 transcription reaction with T7 RNA polymerase 6) pGEM Express Positive control template with Sp6

3.2.3.5 Optimisation of ISH for the detection of HCMV IE-1 mRNA in cells infected *in vitro*

The detection of HCMV IE-1 mRNA by ISH was initially tested on formalin-fixed paraffin-embedded cells infected with HCMV for 4 hrs. The HCMV IE-1 antisense riboprobe is the complementary strand to the mRNA target and was used for detection of HCMV IE-1 gene transcript. The sense probe is the same sequence as the target mRNA and consequently should not bind to the target mRNA. The HCMV IE-1 sense riboprobe was therefore used as a negative control for mRNA detection. ISH was carried out using optimised conditions for HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 riboprobes as described in section 2.4.3.4. However, background staining was high and so conditions for ISH were re-optimised. Acetylation of the tissue sections was incorporated into the pre-treatment steps (see section 2.6.2.1) to help increase specific hybridisation signals and reduce background hybridisation. Non-specific staining was however, still observed, therefore hybridisation conditions were re-tested (see section 2.6.2.2). RNase A digestion for varying times (see section 2.6.2.3) was also incorporated post-hybridisation. RNase A degrades any remaining single stranded RNA probe (which can contribute to background), whereas the double

stranded RNA-RNA hybrids remain as they are RNase resistant. Stringency washes at a range of temp in wash buffer 3 were also tested (see section 2.6.2.3). Optimal positive hybridisation signals were observed with 50 ng riboprobe and when hybridisation was carried out at 42^0C overnight. However, an RNase A (25 µg/ml) digestion step for 30 mins at 37 ^{0}C post-hybridisation was also required to help reduce non-specific staining. Furthermore, stringency washes in wash buffer 3 for 30 mins at 42^0C after RNase A digestion helped improve background staining. Figure 3.26 shows detection of HCMV IE-1 mRNA by ISH on formalin-fixed paraffin-embedded HEL cells infected with HCMV. Specific cytoplasmic hybridisation was only detected when antisense riboprobe was employed (no hybridisation was detected with the sense probe) suggesting RNA is being detected. No hybridisation was detected in the non-infected cells confirming that the HCMV IE-1 antisense riboprobe specifically hybridised to its respective RNA.

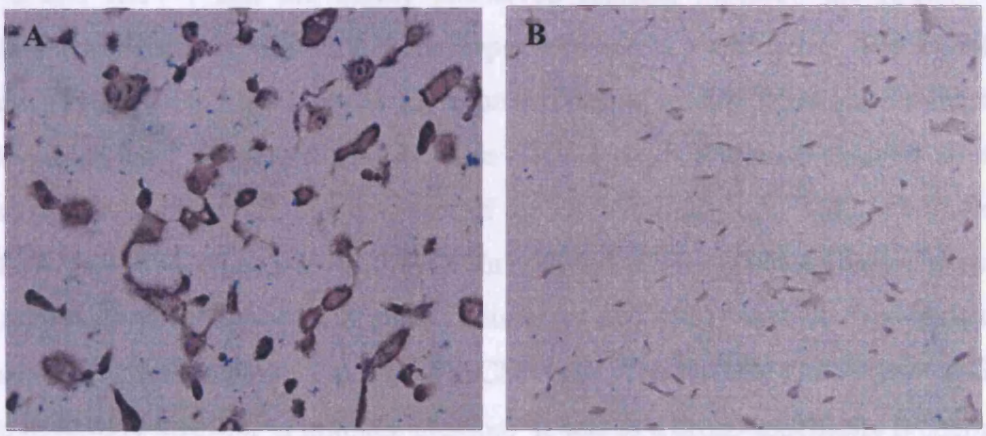


Figure 3.26 Detection of HCMV IE-1 mRNA in formalin-fixed paraffin-embedded MRC-5 cells infected with HCMV. A) Positive cytoplasmic staining was observed with HCMV IE-1 antisense riboprobe. B) No staining with corresponding sense probe.

3.3 Discussion and Conclusions

This chapter describes the development of two methods, ISH and IHC for the detection of HCMV, HHV-6 and HHV-7 infection in formalin-fixed paraffin-embedded cell controls, and the optimisation of the HCMV ISH for detection of viral DNA and in liver and renal biopsies.

Historically, hybridisation was conventionally performed with probes labelled with radioactive isotopes, which follows the detection of sequence specific nucleic acid by autoradiographic analysis. This has now largely been replaced by the use of biotinylated, DIG and electrochemiluminescent labels, facilitating the application of this technique without the use of radioisotopes.

DIG labelled riboprobes specific to HCMV gB, HHV-6 U67 and HHV-7 U42 sequences (Fox *et al.*, 1992; Clark *et al.*, 1996; Kidd *et al.*, 1996) were constructed for ISH. These sequences have been amplified by our diagnostic PCR assays for HCMV, HHV-6 and HHV-7 and are highly conserved regions. HCMV gB (UL55) is an abundant component of the virion envelope, and elicits neutralizing Abs in infected humans. HHV-6 U67 gene codes for a product of unknown function, although it is conserved between variants A and B. The HHV-7 target sequence encodes an amino acid sequence highly homologous to that of the HHV-6 U42 gene. U42 is homologous to ICP27 of HSV (Gompels *et al.*, 1995). In addition, a DIG labelled riboprobe specific to a sequence of HCMV US28 gene (Beisser *et al.*, 2001) was also constructed to compare hybridisation signals to that of HCMV gB. The HCMV US28 gene, a GPCR, has an important function in immune evasion. It acts as a broad specificity β chemokine receptor and can act as a coreceptor for the uptake of HIV (Pleskoff *et al.*, 1997). In addition, it has been demonstrated that the expression of HCMV US28 in SMCs in the presence of MCP-1 and RANTES promoted SMC migration (Streblow *et al.*, 1999). US28-mediated SMC migration thereby provides evidence linking HCMV to the acceleration of vascular diseases (Streblow *et al.*, 1999). ISH using the DIG labelled riboprobes was found to be specific for the detection of HCMV gB, HCMV US28, HHV-6 U67 and HHV-7 U42 DNA in formalin-fixed paraffin-embedded cells infected with respective virus. In order to determine the level of sensitivity of ISH, a riboprobe specific for a 110 bp sequence of human β globin was constructed. The human β globin gene is present at 2 copies per cell, therefore the human β globin sense probe which will

only detect DNA and no mRNA was used to show whether the level of sensitivity of the technique reached single copy levels. No hybridisation signal was observed suggesting that the detection of DNA *in situ* does not approach single-copy levels. Other studies have found ISH detection limits on tissue sections to be in the range of 10-20 copies of mRNA or viral DNA per cell (Hoefler *et al.*, 1986; Speel *et al.*, 1995). Using an EBV-transformed lymphoblastoid cell line with 12-26 copies of integrated HHV-6 genome (V1-LCL), ISH with the HHV-6 U67 riboprobe was also carried out to determine the level of sensitivity of HHV-6 ISH. Although positive hybridisation signals were detected, not all cells stained positive. This is most likely due to the level of viral DNA in the cells being at the limit of the assay's sensitivity. There is no known cell line with integrated HCMV or HHV-7 genome, therefore the sensitivity of ISH with HCMV gB, HCMV US28 and HHV-7 U47 riboprobes could not be determined.

In this chapter, a DIG labelled HCMV IE-1 riboprobe was also constructed for investigating HCMV gene expression. HCMV can establish productive, persistent or latent infections. During a productive infection, expression of HCMV genes occurs in a temporally regulated manner leading to the transcription of IE (α), E (β) and L (γ) genes (Spector *et al.*, 1990). Transcription of the HCMV IE genes is under the control of the major IE promoter (MIEP). This single MIEP controls the expression of two genetic elements, designated IE-1 and IE-2. The IE-1 transcript measures approximately 2.0 kb (Jahn *et al.*, 1984) and the protein is 68-72 kDa (Stinski *et al.*, 1983). The IE-1 genes are the first to be transcribed following entry of the virus into the host cell and their transcription is independent of *de novo* synthesis of viral proteins (LaFemina and Hayward, 1983). Therefore, a riboprobe directed against the IE-1 gene transcript for RNA ISH was used for investigating HCMV in various states of infection in renal allograft biopsies (results described in chapter 4).

Using IHC, the sensitivity and specificity of a range of MAbs for the detection of the human β-herpesvirus proteins in formalin-fixed paraffin-embedded cell controls were evaluated. Three MAbs directed to HCMV proteins characteristic for each temporal stage of replication were used; MAB8126 to HCMV late protein; MAb CCH2 to HCMV p52; and MAB8131 to HCMV IE protein. CCH2 MAb reacted with HCMV infected cells giving an intense nuclear and/or cytoplasmic staining pattern. CCH2 has been found to label "owl's eye" cells and morphologically normal cells in human tissue

sections with widespread HCMV infection (Niedobitek *et al.*, 1988). The epitope of the CCH2 MAb has been mapped to the UL44 reading frame of the HCMV genome (Plachter *et al.*, 1992). Intense cytoplasmic staining was observed in fibroblasts infected with HCMV with MAB8126 to HCMV late protein which is indicative of a productive infection.

The presence of HHV-6-specific proteins was detected in HHV-6B (strain Z29) infected Molt-3 cells using a range of MAbs directed to HHV-6A p41/38 (originally described as detecting both variants), HHV-6B p101 (MAB 8535), HHV-6B gH, and HHV-6 gB. The HHV-6 early protein p41 is encoded by the U27 gene which is conserved in HHV-6A and HHV-6B and shows strong homology with the HCMV UL44 gene coding for the ICP36 family of early-late-class phosphoprotein (Chang and Balachandra, 1991). HHV-6 p41 is a DNA-binding protein and a putative DNA polymerase stimulatory factor (Agulnick *et al.*, 1993). Challoner *et al.* (1995) observed p41 expression using MAb to p41/38 in oligodendrocytes of MS patients but not of controls, thereby suggesting an association of HHV-6 with the etiology or pathogenesis of MS. However, there is data indicating that the two proteins, p41 and p38, recognised by the MAb to p41/38 are not both encoded by the viral genome. The protein p38 is likely to be of cellular origin, since the sequence of a peptide fragment of p38 (Iyengar *et al.*, 1994) shows a strong similarity to human β-actin (protein AAH02409 in GenBank) but lacks homology to the ORFs in HHV-6. Recently, Xu *et al.* (2001) identified the epitope of HHV-6 p41 responsible for the exclusive reactivity of the MAb to p41/38 to a nuclear Ag in HHV-6A but not in HHV-6B. These findings explain the absence of immunohistochemical staining observed with HHV-6 p41/38 in HHV-6B (strain Z29) infected cells. Cytoplasmic staining of HHV-6B p101 structural Ag in HHV-6B infected cells is indicative of an active infection. The 101 kDa apparent molecular mass (101K) virion protein has been identified as the major immunoreactive virion protein of HHV-6B (strain Z29) (Yamamoto *et al.*, 1990; Pellet *et al.*, 1993). The gene encoding the HHV-6B 101K was described as the homolog of HCMV phosphorylated virion tegument protein, pp150. Neipel *et al.* (1992) have identified homology between strongly immunoreactive virion proteins encoded by HCMV and HHV-6A strain U1102, pp150 and p100, respectively.

In the herpesvirus family, several glycoproteins, which are components of the virion envelope, play critical roles in viral infection, including attachment, penetration, and cell-to-cell spread. The amino acid sequence of gB is highly conserved among the members of the herpesvirus family (Pereira, 1994) and gB is essential for virion penetration into cells (Little *et al.*, 1981). gH plays an important role in virion entry and cell-to-cell spread (Babic *et al.*, 1996). Detection of HHV-6 gB and HHV-6B gH Ags were observed in the cytoplasm of Molt-3 cells infected with HHV-6B. MAb to HHV-6B gH was found to be the least sensitive for immunohistochemical detection of formalin-fixed paraffin-embedded Molt-3 cells infected with HHV-6B, whilst MAb to HHV-6 gB gave the best staining intensities with low background levels, therefore MAb to HHV6 gB will be used for the detection of an HHV-6 productive infection in biopsy material.

At present, only MAb 5E1 directed to HHV-7 pp85 is available for the immunological detection of HHV-7 in formalin-fixed paraffin-embedded tissues. MAb 5E1 directed to HHV-7 specific pp85 was sensitive and specific for the detection of formalin-fixed paraffin-embedded Sup T1 cells infected with HHV-7. IHC studies to date have surprisingly not used an isotype control for MAb 5E1 (Kempf *et al.*, 1997; 1998; 2000). Mab 5E1 was therefore subtyped and identified as IgG1. MAb 5E1 does not crossreact with HHV-6 and hence recognizes an HHV-7-specific epitope (Foa-Tomasi *et al.*, 1994; 1996; Stefan *et al.*, 1997). pp85 is encoded by the U14 gene (Stefan *et al.*, 1997), and is a highly immunogenic complex of proteins (Foa-Tomasi *et al.*, 1994; 1996) which localises to the outer layers of the virion tegument (Stefan *et al.*, 1997). As it is directed to a structural component of the virion, a positive reaction is indicative of active viral infection (Stefan *et al.*, 1997).

In summary, the results described in this chapter demonstrate that ISH, and IHC can be used for the detection of HCMV, HHV-6 and HHV-7 DNA and proteins in formalin-fixed paraffin-embedded culture infected cells. More importantly, ISH using a DIG labelled riboprobe specific to HCMV was optimised for the detection of HCMV infected cells in biopsy material. Thus, these methods could now be applied to investigate the involvement of these viruses in disease pathogenesis post-transplant at the tissue level.

Chapter 4 - Role of the human β-herpesviruses in allograft rejection following renal transplantation

4.1 Introduction

Over the past decade, substantial progress has been made in understanding the pathogenesis of HCMV disease after organ allotransplantation. The source of HCMV can be the recipient (reactivation of latent infection) or the donor (causing primary infection if the recipient is HCMV seronegative or reinfection if the recipient is seropositive). Serial measures of HCMV viral load in the blood of recipients of renal (Kidd *et al.*, 2000), liver (Griffiths *et al.*, 1999a) or cardiac transplants demonstrate that HCMV is frequently detected posttransplant at a median of six weeks. In some patients, viral load increases allowing HCMV to cross a putative blood – organ barrier and cause end organ disease manifest as enteritis, hepatitis, retinitis or pneumonitis (Griffiths and Emery, 1997). These are collectively termed the "direct effects" of HCMV because the virus can be seen directly in biopsies of the infected organ using classical histopathological techniques to visualise "owl's eye inclusions" (Smith, 1959; Macasaet *et al.*, 1975). Placebo-controlled trials demonstrate that these direct effects can be prevented through the use of antiviral prophylaxis with GCV (Goodrich *et al.*, 1991; 1993; Winston *et al.*, 1993; Boeckh and Bowden 1995) or VACV (Lowance *et al.*, 1999). Alternatively, patients can be monitored for laboratory evidence of HCMV infection and treated with GCV before disease develops (pre-emptive therapy) (Goodrich *et al.*, 1991; Einsele *et al.*, 1995).

In addition to end-organ disease, HCMV is associated with a variety of other medical conditions (graft rejection, immunosuppression, atherosclerosis) collectively termed "indirect effects" (Rubin, 1989) because the virus is not seen histopathologically in affected organs. Evidence for a causal role for HCMV in these conditions is based upon clinical observations in individual patients, statistical association with HCMV disease and/or HCMV infection in cohorts of patients as well as the results of clinical trials of antiviral agents (Grattan *et al.*, 1989; Loebe *et al.*, 1990; von Willebrand *et al.*, 1986; Fietze *et al.*, 1994; Reinke *et al.*, 1994; Valantine *et al.*, 1999; Rubin *et al.*, 2000). Specifically, the trial of Lowance *et al.* (1999) randomised 208 HCMV seronegative patients to receive prophylaxis with VACV or placebo for ninety days after transplant of a seropositive kidney and reported that the incidence of biopsy-proven acute graft rejection was decreased by 50% among those receiving VACV. This finding, coupled with supporting evidence from animal models of allotransplantation in the presence of

animal CMVs with/without GCV therapy, strongly indicated that CMV was an unrecognized major contributor to acute graft rejections (Lemstrom *et al.*, 1995; 1997).

Evidence against a causal role for HCMV in triggering acute graft rejection comes from several sources. First, not all clinical cohort studies report a statistical association with HCMV (Dickenmann *et al.*, 2001; Sherlock *et al.*, 1991; Teixeira *et al.*, 2000; Boyce *et al.*, 1988). Second, in the Lowance study, a parallel group of 408 renal transplant patients who were HCMV seropositive pre-transplant did not demonstrate a beneficial effect of VACV prophylaxis on the incidence of biopsy-proven graft rejection. Third, other investigations have reported that HHV-6, and/or HHV-7 are also associated with graft rejection and with HCMV end organ disease (Griffiths *et al.*, 1999a; Kidd *et al.*, 2000; Osman *et al.*, 1996; Desjardin *et al.*, 1998; Ratnamohan *et al.*, 1998). HHV-6 and to a lesser extent HHV-7, are susceptible *in vitro* to drugs which inhibit HCMV including GCV (De Clercq *et al.*, 2001). It remains possible therefore that the beneficial effects of antiviral prophylaxis on acute graft rejections may be operating through inhibition of these newer β-herpesviruses and/or inhibition of HCMV.

To address this complex situation QC-PCR assays were previously applied to quantitatively measure the viral load of each of these three β-herpesviruses in transplant patients (Kidd *et al*, 2000; Griffiths *et al.*, 1999a). In the study by Kidd *et al.* (2000) QC-PCR was used to detect active β-herpesvirus infections in the 120 day period following renal transplantation. Detailed clinicopathological analyses were also conducted to reveal symptoms, signs or syndromes associated with these viruses in this setting. HCMV was found to be the most commonly detected virus post-transplant (58% of patients) followed by HHV-7 (46%) and HHV-6 (23%). The median maximum viral loads for HCMV was also significantly higher than those for HHV-6 ($p=0.01$) and HHV-7 ($P>0.0001$), and a trend for HHV-7 viral load to be greater than HHV-6 ($p=0.08$). It was also revealed that in those patients with graft rejection, HHV-7 was associated with more episodes of rejection ($p=0.02$). There was also a significant increase in HCMV disease occurring in patients with HCMV and HHV-7 coinfection compared to those with HCMV infection only ($p=0.04$). Therefore HHV-7 should be further investigated as a possible co-factor in the development of HCMV disease in renal transplant patients and may potentially exacerbate graft rejection. The work in this chapter is an extension of this prospective study, whereby *in situ* techniques

including ISH and IHC (developed in chapter 3) were used to detect these viruses directly in renal biopsies taken from the patients in the original cohort who required biopsy to investigate the cause of graft dysfunction. It is reasoned that, if the *in situ* detection of HCMV was associated statistically with standardised histopathological criteria of rejection in the same biopsies, the hypothesis that HCMV was a cause of biopsy-proven graft rejection would be accepted. In contrast, if HCMV was detectable in biopsies but was not associated with graft rejection, the hypothesis that HCMV infection causes graft dysfunction which mimics rejection clinically would be accepted. Furthermore, the detection of HHV-6 and/or HHV-7 in the biopsies would determine whether these viruses were also involved in the graft rejection process. HCMV gene expression was also investigated in the renal allograft biopsies using a riboprobe directed against the HCMV IE-1 gene transcript for RNA ISH (developed in chapter 3).

In renal (and cardiac) transplantation, high levels of AECAs have been demonstrated in HCMV infected transplant recipients (Toyoda *et al.*, 1997; 1999). These AECA have been proposed to play an important role in the development of humoral allograft rejection after transplantation and could result in reduced long-term allograft survival (Yard *et al.*, 1993; Fredrich *et al.*, 1999). Ab-mediated rejection occurs in approximately 25-50% of biopsy-confirmed acute renal allograft rejection (Lederer *et al.*, 2001; Regele *et al.*, 2001; Mauiyyedi *et al.*, 2002; Nickeleit *et al.*, 2002), has a poorer prognosis than cellular rejection and requires a more aggressive and targeted antirejection therapy (Nickeleit *et al.*, 2002). In recent years the deposition of C4d in the PTC of the renal graft has emerged as an attractive marker of Ab-mediated injury and an important diagnostic criterion (Collins *et al.*, 1999; Koo *et al.*, 2004). In this chapter, evidence of both humoral (using a polyclonal peptide anti-C4d Ab (C4dpAb; Biomedica) (Regele *et al.*, 2001) and cellular graft rejection were also examined because each of these has been associated in different series with HCMV infection.

4.2 Results

4.2.1 Detection of β-herpesvirus DNA by ISH in renal biopsies

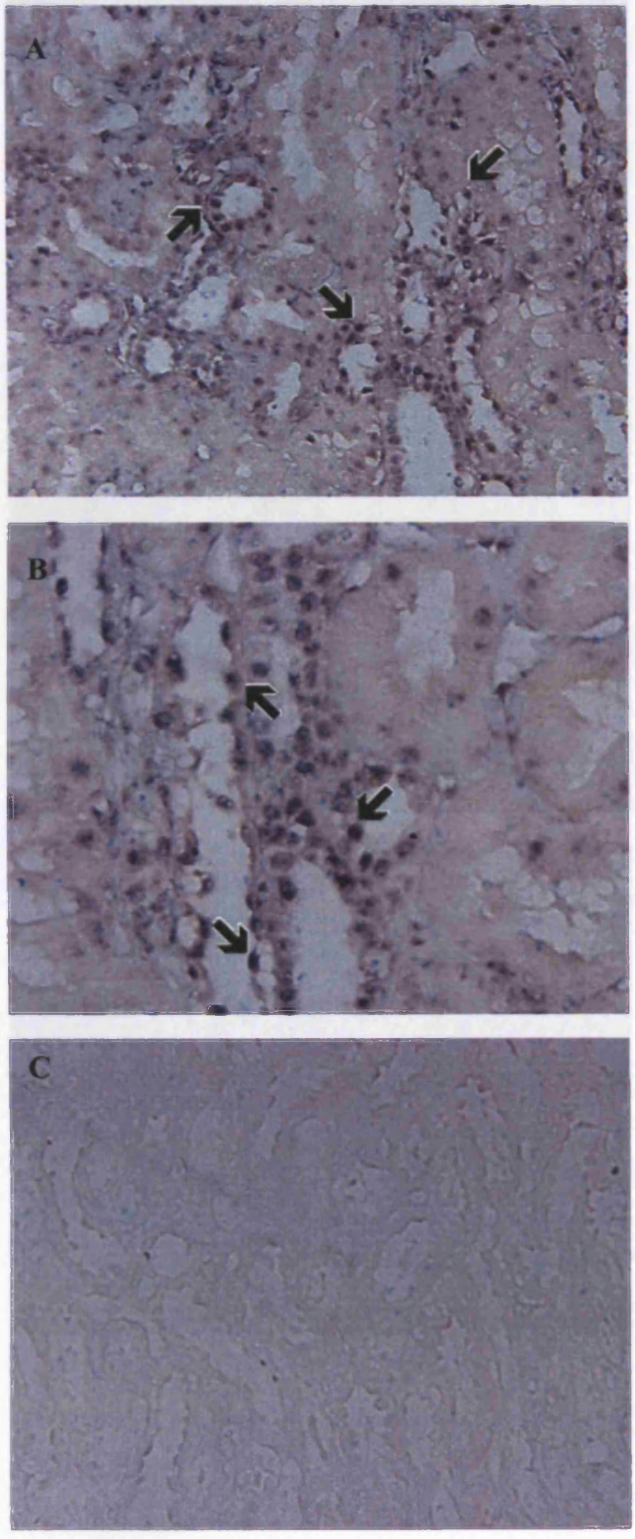
Sixty two renal biopsies from 30 patients obtained from a previous prospective study of 52 patients who underwent their first renal transplantation between 1st August 1993 and 31st January 1995 (Kidd *et al.*, 2000) were available for *in situ* detection of the human β-herpesviruses. In these patients, biopsies were performed at a median of 21 days (range 2 to 107) after transplantation. Two or more biopsies were available from 18 patients, with a median of 2 (range 1 to 5) biopsies. Using optimised ISH conditions for the renal biopsies (see section 2.4.4), each renal sample was stained for HCMV gB, HHV-6 U67 and HHV-7 U42 DNA using DIG labelled riboprobes constructed in section 2.3. HCMV gB DNA was detected in 21/30 (70%) renal transplant patients and 32/62 (52%) biopsies. All renal biopsies from these patients were reviewed by Dr Michael Jarmulowicz, pathologist at the Royal Free and University College Medical School. Table 4.1 shows the detection of HCMV gB DNA by ISH together with the serological status of these patients.

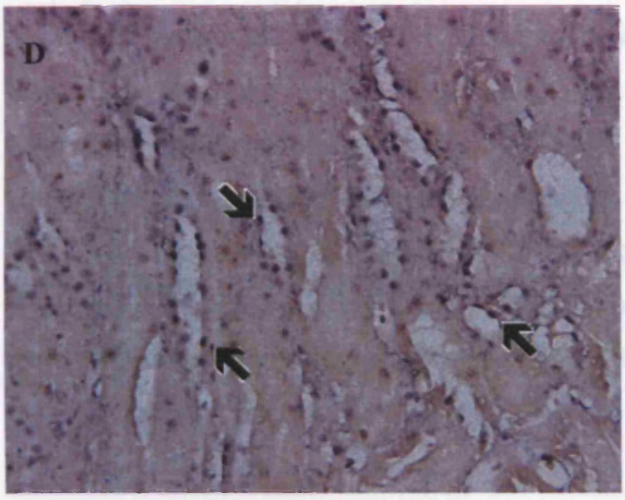
HCMV serostatus	CMV ISH+	CMV ISH-	Total
D+R+	11	2	13
D+R-	3	1	4
D-R+	5	3	8
D-R-	1	2	3
D?R+	1	1	2
	21	9	30

Table 4.1 HCMV serostatus and the detection of HCMV DNA by ISH

HCMV gB DNA was detected in 8 of 12 patients with only one biopsy and in at least one biopsy in 13 of 18 patients with 2 (n =10), or more (n = 8) biopsies. The detection of HCMV in the renal biopsy was frequently seen early during the post-transplantation course. HCMV gB DNA was detected in 19/30 (63%) biopsies taken during 1-19 days (d), in 5/13 (38%) biopsies taken during 20-39 d, and in 8/19 (42%) biopsies taken during 40-120 d post-transplant. Of the 18 patients with 2 or more biopsies, the median time to first HCMV gB DNA positive biopsy by ISH was 19 d (range 5 to 90) post-transplant compared to 32 d (range 2 to 107) post-transplant for HCMV gB DNA negative biopsies. Detection of HCMV gB DNA in the renal biopsies was widespread (Figure 4.1).

Figure 4.1 Renal biopsies from transplant recipients showing detection of HCMV gB DNA by ISH. Patient 1: A and B) HCMV gB DNA and C) No probe. Patient 2: D) and E) HCMV DNA by ISH. Patient 3: F) HCMV DNA by ISH. Arrows indicate positive nuclear staining for HCMV.

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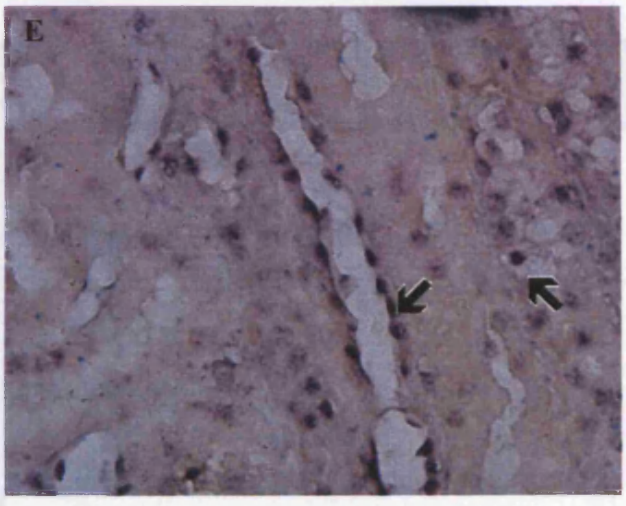
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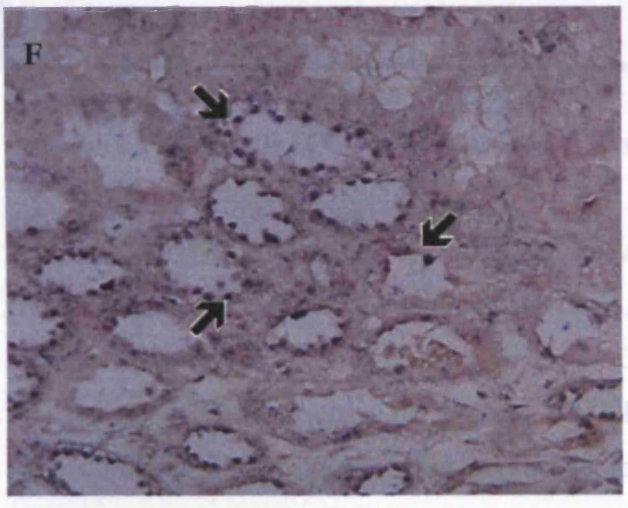
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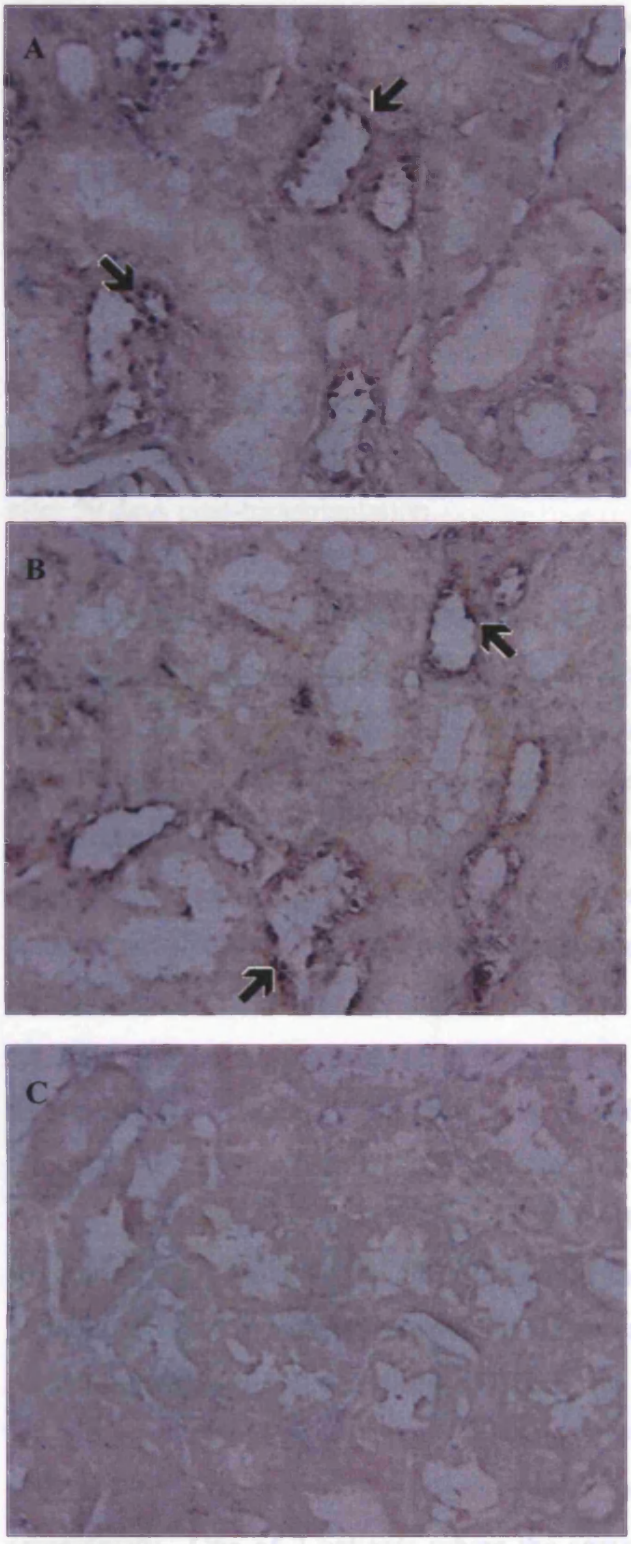
Table 4.2 shows HCMV gB DNA in various structures of the renal allografts. A scoring system for HCMV ISH staining intensities included strong positive staining of HCMV DNA as indicated by +++, moderate staining by ++ and weak staining by + (Dr Michael Jarmulowicz). HCMV gB DNA was found predominantly in the epithelial cells of the distal convoluted tubules (DCT) and the proximal convoluted tubules (PCT) with strong to moderate staining. Endothelial cells also stained positive for HCMV DNA with moderate to slight staining intensities. Other renal structures that stained positive for HCMV gB DNA include the loop of Henle and podocytes, and to a lesser extent mesangial cells. The presence of HHV-6 or HHV-7 DNA was not detected in any of the renal biopsies by ISH.

CMV ISH+ renal structures	no of biopsies	intensity of staining
distal convoluted tubules	32	+++/++
proximal convoluted tubules	13	++/+
podocytes	13	++/+
mesangial cells	3	+
loop of Henle	5	++/+
endothelial cells	5	++/+

Table 4.2 HCMV gB DNA in various renal structures

Ten renal biopsies positive for HCMV gB DNA were also stained for HCMV US28 DNA by ISH to confirm hybridisation results. All 10 HCMV gB DNA positive renal biopsies also stained positive for HCMV US28 DNA. The two gB DNA negative renal biopsies also stained negative for HCMV US28 DNA. The pattern of staining detected for HCMV US28 DNA was very similar to that of HCMV gB DNA, although the intensity of staining for HCMV gB DNA was slightly stronger. Figure 4.2 shows a comparison of positive hybridisation signals for HCMV gB and US28 DNA.

Figure 4.2 Renal biopsy showing comparison of hybridisation signals for HCMV gB and US28 DNA by ISH. A) HCMV gB DNA, B) HCMV US28 DNA and C) negative staining with HHV-6 U67 probe. Arrows indicate positive hybridisation signals.



4.2.2 Correlation of HCMV ISH results with HCMV DNAemia

Twenty of the 30 renal transplant recipients had HCMV DNA detected in the blood by PCR. Of those, 15 (75%) also HCMV DNA detected in their renal biopsies. Eight of those (53%) had at least one biopsy where HCMV DNA was detected within 2 weeks of virus detection in the blood. Of the remaining 7 patients, 3 patients had only one biopsy available, and in all three cases the biopsy was taken more than 2 weeks before HCMV DNA was found in the blood by PCR. In the patients with more than one biopsy, in one patient both biopsies taken on day 8 and day 85 post-transplantation were HCMV DNA positive by ISH and the virus was detected in the blood on day 50 post-transplantation. Two patients had HCMV DNA detected in an initial biopsy, but the virus was not detected in a follow-up biopsy which was taken more than 1 month after virus was detected in the blood. In one patient who had 4 consecutive biopsies, HCMV DNA was detected in 2 biopsies taken on day 5 and day 12, but not in the biopsies taken on days 31 and 35 post-transplantation. HCMV was also detected in the blood of this patient after 59 days post-transplantation.

Of the 21 patients with HCMV DNA detected in at least one of their biopsies, 6 (29%) had no HCMV DNA detected in their blood within 3 months post-transplantation. Of those 6 patients, 4 patients had only one biopsy available, one patient had 3 consecutive biopsies taken on day 45, day 72 and day 90 post-transplantation and virus was detected in the latter two biopsies. Another patient had two biopsies taken on day 2 and day 8 post-transplantation and HCMV DNA was detected in the latter biopsy by ISH. Four patients had no HCMV DNA detected in their biopsies or in blood by PCR.

4.2.3 HCMV serostatus and HCMV disease

Twenty three of the 30 (77%) recipients were HCMV seropositive pretransplantation. Of those, 17 (74%) had HCMV detected in their biopsy by ISH. In 7 HCMV seronegative recipients, 4 (57%) had HCMV gB DNA detected in their biopsy by ISH suggesting virus transmission from the donor kidney. All four patients had poor graft outcome within 5 years post-transplantation and are now deceased. Ten of the 30 (33%) patients developed HCMV disease. This was more common in the HCMV D+/R- serogroup: 3 (75%) of 4 patients developed HCMV disease versus 5 (38%) of 13 patients and 1 (13%) of 8 patients in the D+/R+ and D-/R+ HCMV serogroups, respectively. One of 2 patients where the serogroup status was incomplete developed

CMV disease. Nine of the 10 (90%) patients who developed HCMV disease had HCMV gB DNA detected in at least one of their biopsies by ISH, with all PCR positive in blood. Of 20 patients who did not develop HCMV disease, HCMV gB DNA was detected in the biopsies of 12 (p = .08805).

4.2.4 Correlation of HCMV ISH results with histological rejection

Renal biopsies positive for HCMV gB DNA by ISH were examined to determine whether histological rejection was more frequent in these biopsies. HCMV DNA was detected in 16/30 (53%) biopsies with no rejection or borderline 'suspicious' rejection, and 16/32 (50%) biopsies with acute histological rejection (p = .793). Table 4.3 shows the morphological features of acute rejection in the renal biopsies. In analysis restricted to only those biopsies with acute histological rejection classified by the Banff '93-95' scoring system (Solez *et al.*, 1993; 1996), the detection of HCMV DNA by ISH was also not significantly associated with any type of acute histological rejection (interstitial or vascular), or with any histological parameter attributable to acute allograft rejection including glomerulitis, interstitial inflammation, tubulitis or intimal arteritis.

Histology	All Biopsies	HCMV DNA in renal biopsies		p-value
		Yes	No	
No rejection, *n* (%)	16/62 (28)	7/16 (44)	9/16 (56)	
Borderline rejection, *n* (%)	14/62 (23)	9/14 (64)	5/14 (36)	
Acute rejection, *n* (%)	32/62 (52)	16/32 (50)	16/30 (50)	0.793
Banff 1, *n* (%)	13/27 (48)	7/13 (54)	6/13 (46)	
Banff 2, *n* (%)	14/27 (52)	6/14 (43)	8/14 (57)	0.568
Glomerulitis, *n (%)*	5/37 (14)	1/5 (20)	4/5 (80)	0.169
Interstitial inflammation, *n (%)*	35/37 (95)	18/35 (51)	17/35 (49)	0.257
Tubulitis, *n (%)*	35/37 (95)	17/35 (49)	18/35 (51)	0.987
Intimal arteritis (v1, v2), *n (%)*	14/37 (38)	6/14 (43)	8/14 (57)	0.362

Table 4.3 HCMV DNA by ISH and histopathology

4.2.5 Clinico-pathological analyses

Clinicopathological details of graft and patient survival were available for 28 patients. Patient survival (within 10 yrs post-transplantation) was 64%, of which 12 of 18 patients (67%) had HCMV DNA detected in their graft by ISH. Of the 10 deceased patients, 8 (80%) had HCMV DNA detected in the graft by ISH. One patient died within 1 month post-transplantation with HCMV pneumonitis, and this patient had HCMV DNA detected in the graft and in the blood by ISH and PCR, respectively. It should be emphasized that these patients had biopsies taken because of graft dysfunction, hence the poorer than usual survival. However, the association of HCMV DNA by ISH with patient survival did not reach statistical significance ($p = .268$).

Comparing the detection of HCMV DNA by ISH with graft survival, it was observed that of the 28 patients, only 9 (32%) had grafts that were still functioning up to 10 yrs post-transplantation, of which 5 (55%) had HCMV DNA detected in the graft by ISH. Of the 19 patients with poor graft outcome (patients put on dialysis), 15 (79%) had HCMV DNA detected in the graft by ISH. Although there was a trend towards the detection of HCMV DNA by ISH with poor graft outcome, this did not achieve a statistical significance ($p = .2007$).

4.2.6 C4d staining in allograft biopsies

C4d staining was used to analyse the incidence of acute humoral rejection in the renal transplant patients biopsied during 120d post-transplantation for suspected renal dysfunction. Figure 4.3 shows capillary deposition of complement fragment C4d in graft biopsies. Three types of staining patterns were observed; C4d++ indicates strong widespread staining (involving >50% of interstitial capillaries), C4d+ indicates focal staining, and C4d- absent staining of interstitial capillaries. Twenty nine of 62 (47%) biopsies had C4d-positive staining detected in the endothelial cells of PTC and 6 of these (21%) PTC positive biopsies (10% of all biopsies) also had endothelial staining for C4d in the glomeruli. No biopsy showed C4d glomerular staining in the absence of C4d in PTC. Of the 29 C4d-positive biopsies, strong diffuse C4d staining was found in 10 (34%) of these biopsies (16% of all biopsies).

Nineteen of 30 (63%) transplant patients had at least one biopsy that stained positive for C4d. Of the 19 patients, 12 patients had two (n = 6) or more (n = 6) allograft biopsies. In nine patients, initial C4d staining in endothelial capillaries did not change in follow-up biopsies (6 patients had all C4d negative biopsies and 3 patients had C4d positive staining in both biopsies). In 2 patients, initial biopsy was negative for C4d staining but one or more subsequent biopsies was scored positive (appearance of peritubular C4d deposits). A loss of peritubular C4d staining was observed in 7 patients. Detection of capillary deposition of C4d was mainly during early time course, the median time to first C4d-positive biopsy was 13 (range, 7 to 64) d post-transplantation.

Figure 4.3 Renal biopsy from a patient showing diffuse C4d staining in endothelial cells. A) peritubular capillaries B) glomeruli. C) IgG control.

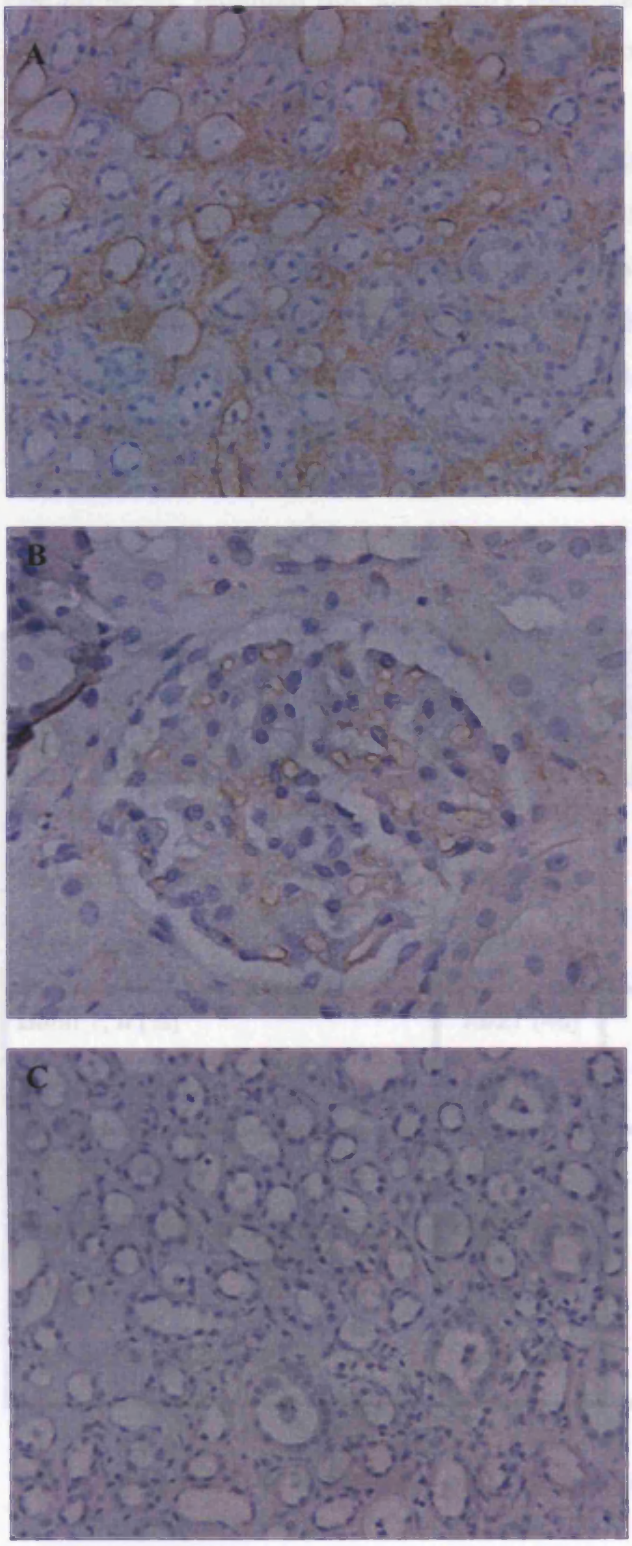


Table 4.4 shows C4d deposition and histopathology. In biopsies with peritubular C4d deposits, acute rejection was more common (18/29, 62%) than in C4d negative biopsies (14/33, 42%), although not statistically significant (p = .1225). In biopsies with C4d positive staining, Banff type II (vascular) rejection was more common than Banff type I (interstitial) rejection (p = 0.034). A statistical association between C4d staining and intimal arteritis (p = .0045), characteristic of type II rejection was also found. No significant association was found between C4d staining and other histological features of acute rejection including glomerulitis, interstitial inflammation, and tubulitis. In biopsies with peritubular C4d deposits, the detection of HCMV DNA was more common (18/29, 62%) than in C4d- biopsies (14/33, 42%) although this difference was not statistically significant (p = .1225).

	All Biopsies	C4d in PTC		p-value
		Yes	No	
No rejection, *n* (%)	16/62 (28)	6/16 (38)	10/16 (63)	
Borderline rejection, *n* (%)	14/62 (23)	5/14 (36)	9/14 (64)	
Acute rejection, *n* (%)	32/62 (52)	18/32 (56)	14/32 (44)	0.123
Banff 1, *n* (%)	13/27 (48)	5/13 (38)	8/13 (62)	
Banff 2, *n* (%)	14/27 (52)	11/14 (79)	3/14 (21)	0.034
Glomerulitis, *n (%)*	5/37 (14)	3/5 (60)	2/5 (40)	0.585
Interstitial inflammation, *n (%)*	35/37 (95)	16/35 (46)	19/35 (54)	0.135
Tubulitis, *n (%)*	35/37 (95)	16/35 (46)	19/35 (54)	0.135
Intimal arteritis (v1, v2), *n (%)*	14/37 (38)	11/14 (79)	3/14 (21)	0.0045

Table 4.4 shows C4d deposition and histopathology.

4.2.7 Detection of HCMV specific proteins by IHC

Three MAbs (as described in section 2.5.6) were used to detect HCMV-specific proteins expressed at different stages of replication. There was no detection of the three HCMV Ags by IHC in the 10 renal biopsies which had tested positive for HCMV DNA by ISH.

4.2.8 Detection of active caspase 3 by IHC

Evidence of apoptosis was also investigated in renal biopsies infected with HCMV DNA using a MAb directed to the active form of caspase-3 (Pharmingen). Active caspase-3 was observed in both induced and non-induced HL60 cells (positive controls). Approximately 50-60% of etoposide treated HL-60 cells showed active caspase-3 staining most of them with clear apoptotic morphology. In non-induced cells only a few positive cells were detected. Figure 4.4 shows nuclear and/or cytoplasmic staining of active caspase-3 in HL60 cells treated with etoposide. Active caspase-3 staining was not found in any of the 10 renal biopsies tested. One renal biopsy showed a few active caspase 3-positive cells, as indicated by cytoplasmic and/or nuclear staining. However, the number of positively stained cells was less than 5% and so was scored negative (see figure 4.5).

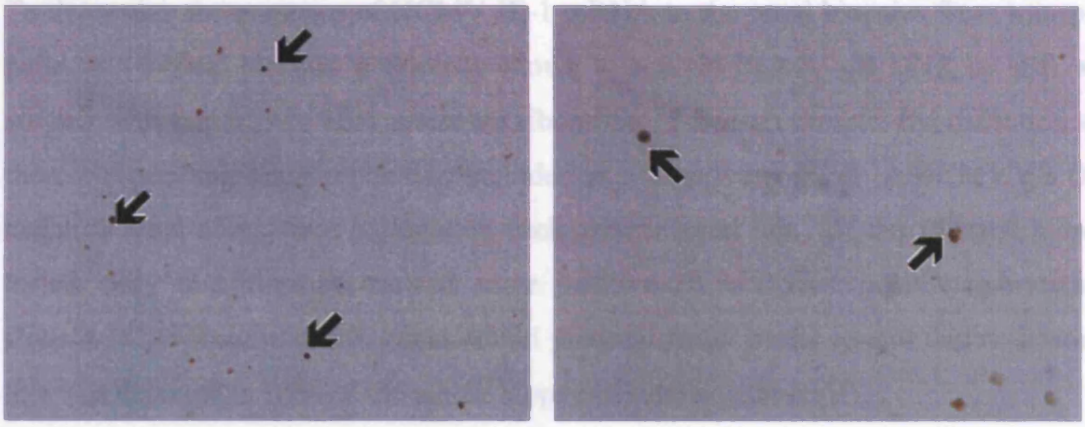


Figure 4.4 Detection of active caspase-3 in etoposide treated HL60 cells. Arrows indicate brown nuclear localised staining and/or cytoplasmic staining of active caspase-3 positive cells.

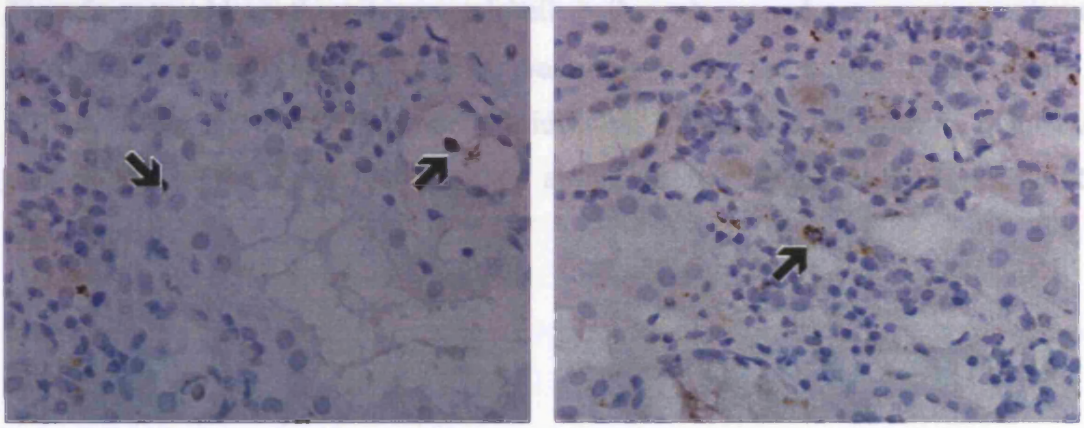


Figure 4.5 Apoptosis in a HCMV gB DNA positive renal biopsy. Only a few cells stained positive for active caspase-3 (see arrows).

4.2.9 ISH with HCMV IE-1 antisense riboprobe on renal biopsies

To determine the presence of HCMV IE-1 mRNA in the renal biopsies from transplant patients, 10 renal biopsies previously shown to contain HCMV gB DNA by ISH were stained with the HCMV IE-1 antisense riboprobe. For each sample, hybridisation with the corresponding sense probe was included as a negative control. A HCMV gB DNA negative renal biopsy was included in each experimental run. Of the 10 renal biopsies tested, only two biopsies showed some positive HCMV IE-1 mRNA hybridisation signals. Cytoplasmic staining was found predominantly in the renal tubules, however, this was detected in <5% of the whole biopsy (results not shown).

The lack of positive hybridisation signals detected in the renal biopsies with the HCMV IE-1 antisense riboprobe maybe due to either low levels of HCMV IE-1 gene transcript in these biopsies which are not detectable by ISH, or it may reflect a problem with tissue preparation or the tissue itself or the technique. If the quality of the tissue is poor and RNA is degraded it will be very difficult to obtain satisfactory results with ISH. Therefore, to address these issues, a labelled riboprobe complementary to an abundant "housekeeping gene" such as β-actin was used to test the quality of the tissue and mRNA within the tissue. Positive hybridisation signals will be detected if mRNA is present within the tissue, whereas an absent or low signal suggests tissue RNA degradation.

4.2.10 ISH with β-actin antisense riboprobe (Roche) on 4% paraformaldehyde-fixed human cell suspensions

ISH with the β-actin antisense riboprobe was initially carried out on HEL cell suspensions that had been fixed in 4% paraformaldehyde for 20 mins at 4^0C. These cells were fixed for a short period of time to minimize mRNA degradation. The ISH conditions used were the same as that described in section 2.3.1. HEL cells treated with RNase were included as a negative control to confirm that the hybridisation signals were mRNA specific. Positive hybridisation signals were detected on these cells (results not shown).

4.2.11 ISH with β-actin antisense riboprobe (Roche) on formalin-fixed paraffin-embedded human cell suspensions

ISH with the β-actin antisense riboprobe was then carried out on formalin-fixed paraffin-embedded HEL cells. Hybridisation was carried out at 42^0C overnight with 50 ng, 25 ng and 5 ng probe. HEL cells treated with RNAse A (0.1 and 1 mg/ml), and cells treated with DNAse I (0.1U/μl) as described in section 2.6.4.2 were also included to confirm the specificity of the reaction. RNAsin was also incorporated into the hybridisation mix to further inhibit any RNases which may degrade the riboprobe. Positive hybridisation signals were detected with this probe at all concentrations tested, with stronger signals detected as concentration of probe increased (see figure 4.6). Positive hybridisation signals were detected when cells were treated with DNase (degrades DNA), but not when cells were treated with RNase (0.1 or 1 mg/ml) confirming that hybridisation is RNA specific.

4.2.12 ISH with β-actin antisense riboprobe on formalin-fixed paraffin-embedded renal biopsies

Five renal biopsies were initially tested with β-actin antisense riboprobe using the same *in situ* hybridisation method as described in section 2.4.4. Results were difficult to interpret as background staining was very high. To help reduce background levels an acetic anhydride step was incorporated during pre-treatment of sections (see section 2.6.2.1). Hybridisation using a lower concentration of probe (5 ng per reaction) was also carried out at 42^0C overnight. Ten renal biopsies (5 HCMV gB DNA positive biopsies from section 4.2.1, and 5 HCMV gB DNA negative renal biopsies) were again tested. For each sample, a negative control consisting of the same sample treated with RNAse was run in parallel to ensure hybridisation signals were mRNA specific. Non-specific background staining was still too high for five biopsies. Two biopsies showed positive mRNA hybridisation signals. Detection of mRNA was not uniform, but more localised in the renal biopsy (see figure 4.7). mRNA was predominantly detected in the renal tubules. RNase treated biopsies stained negative indicating hybridisation to be RNA specific. Three biopsies showed very little mRNA detection, if any.

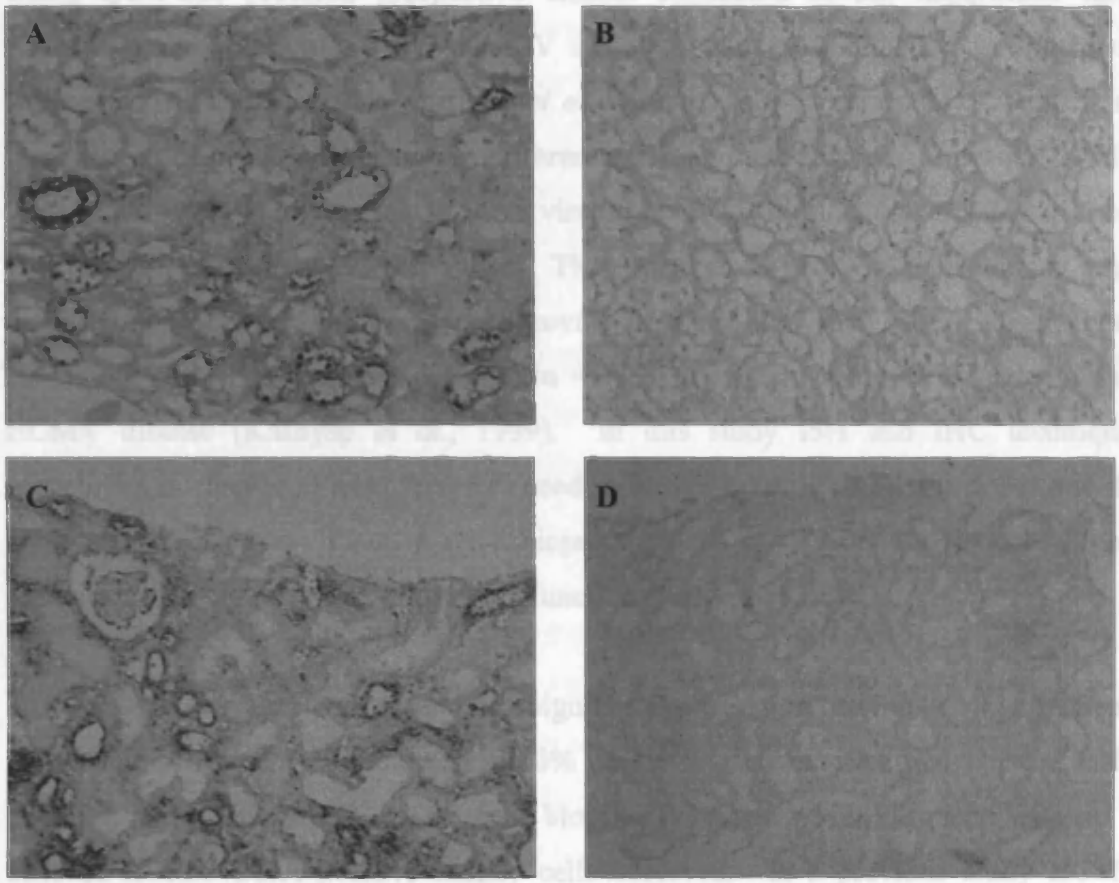


Figure 4.7 Detection of human β-actin mRNA in renal biopsies by ISH. Patient 1: A) Positive human β-actin mRNA hybridisation and B) no staining in the same section treated with RNase. Patient 2: C) Positive β-actin mRNA hybridisation and B) shows no staining in the same section treated with RNAse.

4.3 Discussion and Conclusions

Using QC-PCR, previous prospective studies conducted in our department have reported an association between HCMV infection and graft rejection in transplant recipients (Kidd *et al.*, 2000; Griffiths *et al.*, 1999a). HHV-6 and HHV-7 infections were also linked to graft rejection in different transplant populations. However to fully understand the pathological role of these viruses in allograft rejection detection of these viruses must be made at the tissue level. The occurrence of HCMV inclusions in renal tissue examined by light microscopy provide direct evidence of tissue invasiveness. However, these inclusions are detected in <1% of transplant biopsies of patients with HCMV disease (Kashyap *et al.*, 1999). In this study ISH and IHC techniques (developed in chapter 3) were therefore used to further investigate the incidence and the pathogenesis of these viruses in renal biopsies taken from a previous cohort of renal transplant patients suspected of graft dysfunction (Kidd *et al.*, 2000).

HCMV DNA was detected by ISH in a significant proportion of biopsies from patients with renal allografts. Approximately 50% of these renal biopsies had HCMV DNA detected, and within a majority of these biopsies detection was widespread with up to 50% of HCMV DNA positive kidney cells observed. In a previous study HCMV genomes were documented in up to 41% of routine allograft biopsies by ISH technique (Ulrich *et al.*, 1986). The biopsies containing HCMV DNA were not more likely to show graft rejection so the hypothesis that HCMV is a frequent trigger of rejection was rejected. This finding is similar to another study where *in situ* detection of HCMV was not found to be significantly associated with acute rejection (Andersen *et al.*, 1990). Instead, it is proposed that CMV infection of the graft causes post transplant graft dysfunction which mimics rejection clinically and which can be prevented by antiviral prophylaxis such as that used by Lowance *et al.* (1999). In support of this conclusion, in most of our cases the donor had serological evidence of past HCMV infection consistent with the donor origin of HCMV exactly as seen by Lowance *et al.* (1999). Since no biopsy contained HHV-6 or HHV-7 DNA using ISH of similar sensitivity the role of these β–herpesviruses in graft rejection or dysfunction is also rejected. This finding is in contrast to the situation in the blood where HHV-7 DNA was detected by PCR in these patients with rejection. HHV-7 was also associated with increased episodes of rejection (Kidd *et al.*, 2000).

Several studies have demonstrated that various cell types in the kidney, including mesangial cells, glomerular epithelial cells, endothelial cells and tubular epithelial cells can be infected by HCMV *in vitro* (Ustinov *et al.*, 1991; Heieren *et al.*, 1988a; 1988b) and *in vivo* (Payton *et al.*, 1987; Bissinger *et al.*, 2002; Hendrix *et al.*, 1997). In the present study, HCMV DNA was also found to be widely distributed in a variety of cell types, although tubular epithelial cells were most frequently positive, this finding is similar to other studies (Ulrich *et al.*, 1986; Hendrix *et al.*, 1997).

Results in this chapter suggest a trend towards the presence of HCMV DNA in the graft as detected by ISH and the long-term outcome among the renal transplant recipients, however, this did not achieve statistical significance which is a reflection of low patient numbers.

In this chapter ISH was used to detect the presence of β-herpesvirus DNA in renal allografts using riboprobes specific for HCMV, HHV-6 and HHV-7. A riboprobe directed to HCMV IE-1 gene transcript (developed in chapter 3) was also used to further examine for HCMV gene expression in these biopsies. HCMV IE-1 gene transcripts were not detected in the renal allografts by RNA ISH. β-actin transcripts were only detected in some renal allografts. β-actin mRNA in these biopsies were not evenly distributed but focal and random. These results suggest that the renal allografts may not be suitable for looking at mRNA (and therefore no HCMV IE-1 gene transcripts) since RNA degradation is likely to have occurred resulting in no, or low abundance of β-actin transcripts detected. Therefore, although HCMV gB DNA was found in the renal allografts, the type of HCMV infection could not be established by ISH. To further discriminate between a latent or productive infection, IHC was utilised to identify for viral Ags. The failure to detect HCMV Ags shows that HCMV expression was at a low level and explains why this phenomenon has not been described previously by histopathologists using IHC or examination for owl's eye inclusions which are known to be insensitive (Mattes *et al.*, 2000; Smith *et al.*, 1975). The sensitivity of the ISH technique is unlikely to detect latent infection, so the HCMV DNA in the biopsies most likely represents low level virus replication or abortive infection.

Immunohistochemical staining with an anti-active caspase 3 mAb failed to identify any evidence of apoptosis in HCMV DNA positive biopsies. Apoptosis is an important innate antiviral defence that can result in aborted infection and the elimination of virus-infected cells. However, despite the presence of extensive HCMV DNA in the graft, apoptosis is not occurring. HCMV has been found to encode four anti-apoptotic gene products, IE-1, IE-2 pUL36 and pUL37. The IE-1 and IE-2 proteins each inhibit the induction of apoptosis by TNF-α or by the E1B-19-kDa-protein-deficient adenovirus (Zhu *et al.*, 1995). UL36 encodes a protein designated vICA (viral inhibitor of caspase activation) which inhibit caspase-8 activation (Skaletskaya *et al.*, 2001) and UL37 encodes a protein designated vMIA (viral mitochondria-localized inhibitor of apoptosis) which inhibits mitochondrial megapore activation in a manner similar to members of the anti-apoptotic *Bcl* family (Goldmacher *et al.*, 1999).

Studies have shown that the onset of HCMV infection, as indicated by the detection of HCMV in the blood by PCR is around 4 to 8 weeks after SOT (Kidd *et al.*, 2000; Griffiths *et al.*, 1999a). Although the exact time course of HCMV in renal allografts can only be determined in a prospective study testing protocol biopsies, detection of HCMV DNA by ISH was found predominantly during the early post-transplantation course (63% biopsies taken during 1-19 d post-transplantation were HCMV positive) suggesting that in some cases it is most likely to be of donor origin. The detection of HCMV DNA in a biopsy by ISH frequently preceded detection of virus in the blood. Twenty percent of patients had at least one HCMV DNA positive renal biopsy, without detection of HCMV in the peripheral blood in the 120 d post-transplant period. Although no detectable HCMV DNA in the blood does not rule out the presence of low levels of active infection below the detection level of our PCR, the findings may reflect the role of local immune response in limiting virus dissemination following reactivation. Evidence from mouse cytomegalovirus (MCMV) models have also demonstrated the importance of the immune response in limiting reactivation at local sites where it occurs, and preventing dissemination of virus to cause widespread infection (Reddehase *et al.*, 2000).

Over the years evidence has been accumulating to indicate that acute rejection is not exclusively a T-cell mediated process but may also involve the generation of circulating Abs that react against the graft. Until recently, the detection of Ab-mediated rejection

has been difficult to diagnose histologically due to the rapid removal of Igs from endothelial cell surfaces. It was work first pioneered by Feucht *et al.* (1991; 1993) and later demonstrated in subsequent studies (Collins *et al.*, 1999; Regele *et al.*, 2001) that an end-product of complement C4d in PTC represented an attractive marker for acute humoral rejection. In the present study, C4d staining was therefore used to analyse the incidence of acute humoral rejection in the renal transplant patients biopsied during 120d post-transplantation for suspected renal dysfunction. Approximately 50% of biopsies showed C4d staining, this finding is similar to that of other studies (Lederer *et al.*, 2001; Feucht *et al.*, 1993). In the present study both diffuse and focal staining of interstitial capillaries were included as "positive for humoral rejection" (similar to the approach used by Lederer *et al.*, 2001). Feucht *et al.* (1993) demonstrated in a previous study that the appearance of capillary C4d can follow a dynamic course. Therefore, the staining of few capillaries indicates a transitional state, either representing an ongoing attack (resulting later in diffuse staining) or the 'wash-out phase' of a previous attack (Lederer *et al.*, 2001; Regele *et al.*, 2002). Focal staining is therefore clinically relevant (Feucht *et al.*, 1993; Lederer *et al.*, 2001). Other studies have reported a much lower number of C4d-positive biopsies (30%) and this may in part be due to the inclusion of only widespread and bright C4d capillary staining (Mauiyyedi *et al.*, 2002). Other technical considerations may also contribute, such as sensitivity of the technique used (e.g. IHC or indirect immunofluorescence), Ab type and titre.

In some studies accumulation of C4d in renal allografts was found to be associated with acute cellular rejection (Feucht *et al.*, 1993; Nickeleit *et al.*, 2002), whereas other studies have found PTC C4d staining to be independent of histologic rejection type (Regele *et al.*, 2001; Herzenberg *et al.*, 2002). In this study endothelial C4d staining was associated with intimal arteritis, a morphological sign of acute cellular rejection, this finding is similar to that of Nickeleit *et al.* (2002). Endothelial C4d staining has also been found to be associated with vascular (Banff type II) rejection. Another study by Feucht *et al.* (1993) also reported a close correlation between PTC C4d deposition and vascular rejection. Endothelial C4d deposition was also demonstrated in a number of biopsies without morphological signs of cellular rejection. This finding is in line with recent studies showing that Ab-mediated rejection can occur in the absence of cellular rejection (Trpkov *et al.*, 1996; Regele *et al.*, 2001). It has been suggested that

humoral mediated injury without signs of cellular rejection represents pure Ab-mediated rejection (Collins *et al.*, 1999).

In some transplant recipients with HCMV infections, high levels of AECA have been found, and these Abs have been suggested to play an important role in the development of Ab-mediated rejection (Yard *et al.*, 1993; Fredrich *et al.*, 1999). However, in a recent study by Nickeleit *et al.* (2002), clinical evidence of infections within 2 weeks before a renal allograft biopsy (HCMV, VZV, sepsis, bacterial infections) was not significantly associated with C4d deposition (Nickeleit *et al.*, 2002). In this study, the detection of HCMV DNA in the renal biopsies by ISH was not associated with C4d deposition.

As well as aiding understanding of pathogenesis of graft rejection, the findings of this study have important clinical implications. A common clinical conundrum in patients who present with fever and graft dysfunction is the differential diagnosis of rejection *or* infection. The finding that HCMV is frequently detected in biopsies shows that rejection *and* infection frequently co-exist. The *in situ* hybridisation method for HCMV could be used to test biopsies collected during clinical trials allowing patients to be randomised to receive GCV or placebo; the results would then determine whether patients with graft dysfunction should be given anti HCMV therapy in addition to treatment for their graft rejection.

Chapter 5 – Role of the human β-herpesviruses in allograft rejection following liver transplantation

5.1 Introduction

HCMV is recognized as the single most important infectious agent post-transplantation, causing severe end-organ diseases. HCMV has also been associated with acute and chronic rejection of solid organs, or GVHD in the case of BMT, and secondary bacterial and fungal infections. Following liver transplantation HCMV infection occurs in 30-65% of patients, of which 18-40% were symptomatic infection, and mostly developed one to three months after transplantation (Gao *et al.*, 2004). The evidence for association of CMV with an increased risk of acute graft rejection comes from several cohort studies in liver (Fietze *et al.*, 1994), renal (von Willebrand *et al.*, 1986; Reinke *et al.*, 1994) and heart (Grattan *et al.*, 1989; Loebe *et al.*, 1990) transplant recipients. In liver transplantation the importance of concomitant CMV infection during late acute rejections has been reported (Cakaloglu *et al.*, 1995). Persistent HCMV DNA has been demonstrated in hepatocytes of liver grafts with VBDS (Arnold *et al.*, 1992), in bile ducts and vascular structures of liver allografts associated with chronic rejection (Lautenschlager *et al.*, 1997a). Various animal models have also been developed to study the effect of CMV infection on organ allografts. In the rat, CMV has been shown to increase inflammation, cause vascular disease, and accelerate chronic rejection in kidney and aortic allografts (Lautenschlager *et al.*, 1997b; Lemstrom *et al.*, 1993; 1995; Streblow *et al.*, 2003). In a rat liver allograft model, CMV was found to significantly increase portal inflammation and cause more severe bile duct damage in liver allografts undergoing acute rejection, than in the uninfected grafts. CMV was also linked to the induction of VCAM-1 in the endothelial cells. The results support an association between CMV infection and the immunological mechanisms of rejection, as well as the role of CMV in the development of bile duct damage in liver allografts (Martelius *et al.*, 1998). In studies involving antiviral therapy for CMV, treatment of CMV-infected graft recipients with GCV resulted in the elimination of virus-induced transplant vascular sclerosis (TVS) and prolonged graft survival (Lemstrom *et al.*, 1997; Lemstrom *et al.*, 1994). In a prospective study of heart transplants, treatment with prophylactic GCV delayed the time to allograft rejection (Valantine *et al.*, 1999), thereby implicating CMV as a causative agent of chronic rejection.

Evidence against a causal role of CMV in graft rejection also exists. Several clinical cohort studies have not reported a statistical association between HCMV and acute graft rejection (Sherlock *et al.*, 1991; Teixeira *et al.*, 2000; Boyce *et al.*, 1988). In a prospective study by Paya *et al.* (1992) a correlation between the occurrence of HCMV infection and VBDS in liver allograft recipients was not found. Long term complications were also not observed in a large study on CMV hepatitis, and an association with chronic rejection was also not found (Seehofer *et al.*, 2002), therefore the causative link still remains questionable. Immunosuppression associated with transplantation may also enhance the replication of other β-herpesviruses, namely HHV- 6, and/or HHV-7. In liver transplant recipients, concurrent HHV-6 and HHV-7 reactivation is frequently found together with HCMV (Lautenschlager *et al.*, 2002; Razonable and Paya, 2002b). Several studies have shown that coinfection of HHV-6 and/or HHV-7 with HCMV is associated with an increase risk of HCMV disease in transplant recipients (Dockrell *et al.*, 1997; Mendez *et al.*, 2001; Desjardin *et al.*, 1998; 2001; Tong *et al.*, 2000). These viruses have also been associated with graft rejection after transplantation (Lautenschlager *et al.*, 1998; Lautenschlager *et al.*, 2000; Humar *et al.*, 2002; Griffiths *et al.*, 1999a; Kidd *et al.*, 2000). Other studies however, have not been able to identify specific disease associations with HHV-6 infection in liver or renal transplant recipients (Schmidt *et al.*, 1996; Herbein *et al.*, 1996).

In prospective studies conducted in our department a relationship between HHV-6 and HHV-7 and graft rejection in different organ transplant recipients has been suggested (Griifiths *et al.*, 1999a; Kidd *et al.*, 2000). In the study by Griffiths *et al.* (1999a), qualitative PCR was used to detect active β-herpesvirus infections in the 120 day period following liver transplantation. Infections with HHV-6 and HHV-7 were found to be common after transplantation, and they occurred earlier than HCMV infection (the median time to first PCR-positive sample was 20 days for HHV-6, 26 days for HHV-7 and 36 days for HCMV). QC-PCR results showed that the median peak virus load for HCMV was significantly greater than that for HHV-6 and HHV-7. Clinicopathological analyses also revealed that all three viruses were temporally associated with altered liver function, suggesting each virus was associated with episodes of hepatitis. HCMV and HHV-6, but not HHV-7 were also independently associated with biopsy proven graft rejection. It was therefore concluded that HHV-6 may be a previously unrecognized pathogen in this patient group (Griffiths *et al.*, 1999a).

The work in this chapter is an extension of this prospective study whereby ISH and IHC were used for the direct detection of these viruses in liver biopsies taken from the patients in the original cohort. Protocolled liver biopsies were obtained at scheduled times after transplantation, and whenever liver dysfunction occurred (Griffiths *et al.*, 1999a). Since it was not feasible to test all biopsies, biopsies were selected from patients taken nearest to the time of PCR positivity in the blood for any of the human β-herpesviruses. The detection of HCMV, HHV-6 and/or HHV-7 in the biopsies would determine whether these viruses were involved in the graft rejection process. ISH with DIG labelled riboprobes specific for HCMV, HHV-6, and HHV-7 were used for detection of virus DNA in the liver biopsies. IHC with MAbs directed against human β-herpesvirus late proteins; HCMV late protein, HHV-6 gB, and HHV-7 pp85, were used to detect active infections.

5.2 Results

5.2.1 Detection of human β-herpesviruses DNA in liver biopsies by ISH

Fifty four allograft biopsies from 30 patients obtained from a previous prospective study of 60 patients who underwent their first liver transplantation between 1st August 1993 to 11th October 1995 (Griffiths *et al.*, 1999a) were available for *in situ* detection of the human β-herpesviruses. Inclusion criteria for this present study was the availability of at least one fine needle biopsy taken near the time of PCR positivity in blood for any of the human β-herpesviruses (median –2.5, range -33 to +8 days) during the 120-day post-transplant period. Consecutive liver biopsies were also taken for 4 patients. Using optimised ISH conditions for the liver biopsies (see section 2.4.3.4), each liver sample was stained for HCMV gB, HHV-6 U67 and HHV-7 DNA using DIG labelled riboprobes constructed in section 2.2. Two biopsies from 2 patients were insufficient for ISH. HCMV gB DNA was detected in 17/29 (59%) liver transplant patients and 28/52 (54%) biopsies by ISH. No CMV inclusion bodies were demonstrated in any of these liver biopsies. One patient had insufficient biopsy for ISH. All liver biopsies from these patients were reviewed by Dr Richard Standish, Department of Histopathology, Royal Free and University College Medical School. Table 5.1 shows the detection of HCMV gB DNA by ISH together with the serological status of these patients.

Table 5.1 HCMV serostatus and the detection of HCMV gB DNA by ISH

HCMV serostatus	CMV ISH+	CMV ISH-	Total
D+R+	7	3	10
D+R-	3	4	7
D-R+	3	4	7
D-R-	2	0	2
D-R?	1	0	1
D+R?	1	1	2

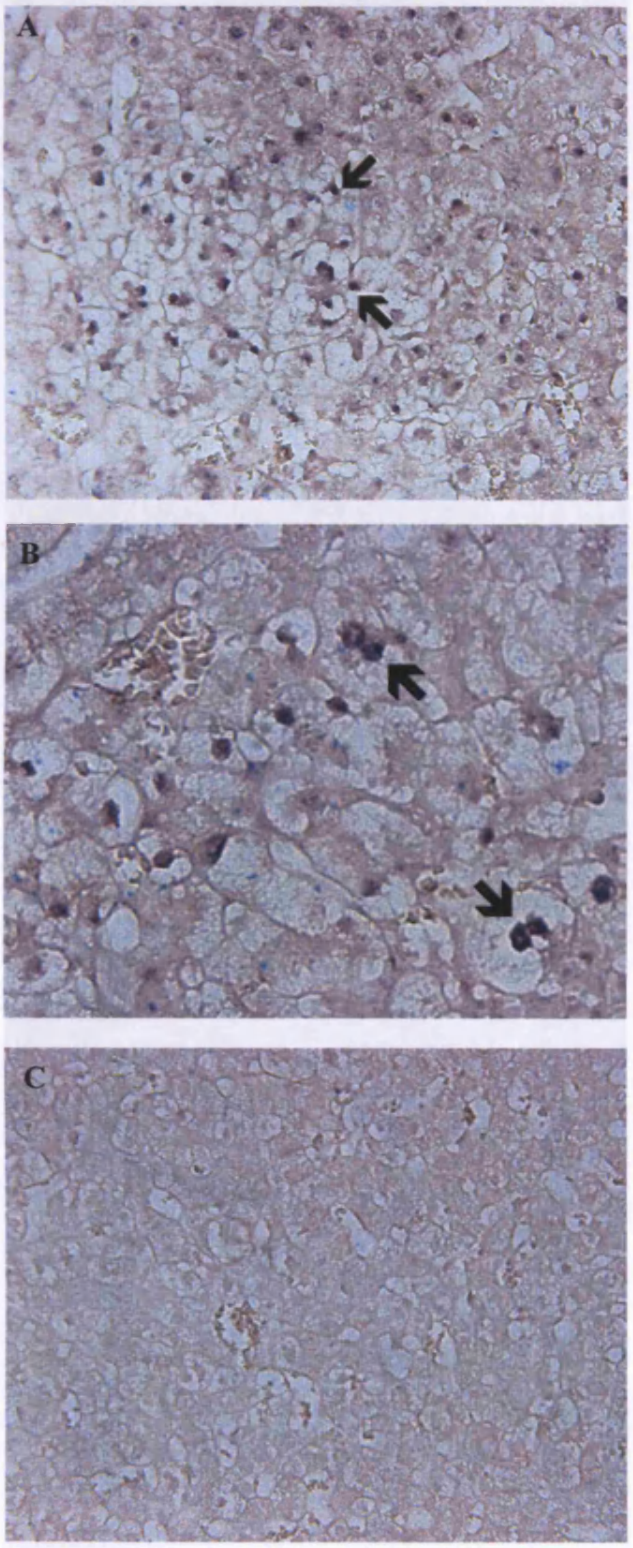
HCMV gB DNA was detected in various structures of the liver allograft (see table 5.2). HCMV DNA was found predominantly in the nuclei of liver hepatocytes, and endothelial cells. Sinusoidal endothelial cells and to a lesser extent Kupffer cells and inflammatory cells also stained positive for HCMV gB DNA. The number of HCMV gB DNA-positive hepatocytes varied between liver biopsies. In some, none or only a few occasional hepatocyte nuclei were positive for HCMV gB DNA, whereas in other liver biopsies infected hepatocytes were common (see fig 5.1). The intensity of HCMV gB DNA staining also varied. However, the distribution and intensity of staining of infected cells in the liver biopsy did not correlate with histological rejection. Staining for HHV-6 and HHV-7 DNA by ISH did not produce convincing results.

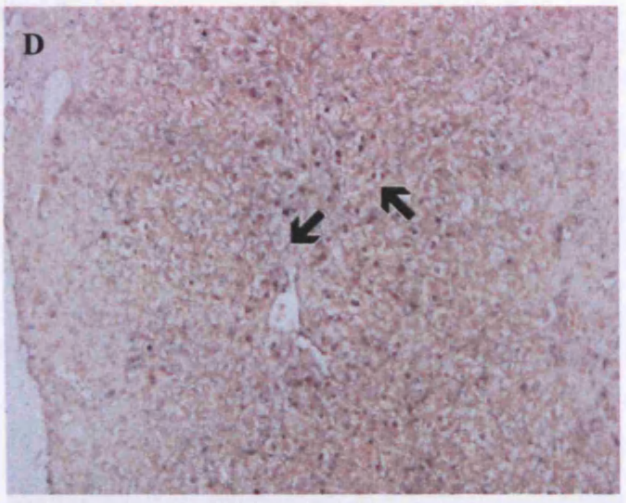
Ten liver biopsies positive for HCMV gB DNA were also stained for HCMV US28 DNA by ISH to confirm hybridisation results. All 10 HCMV gB DNA positive liver biopsies also stained positive for HCMV US28 DNA. Five HCMV gB DNA negative liver biopsies also stained negative for HCMV US28 DNA. The pattern of staining detected for HCMV US28 DNA was very similar to that of HCMV gB DNA, with positive hybridisation signals predominantly detected in the nuclei of liver hepatocytes. Figure 5.2 shows a comparison of positive hybridisation signals for HCMV gB and US28 DNA.

Table 5.2 HCMV gB DNA in various structures of the liver

Liver biopsy no	HCMV DNA in the liver				
	hepatocytes	endothelial cells	sinusoidal endothelial	Kupffer cells	inflammatory cells
1	++	++			+
2	++	++		++	
3	++				
4	++	++	++		
5	+				
6	+	+			
7	+				
8	+				
9	++				
10	+				
11	+				
12	++				
13	+		++	+	
14	+				
15	+				
16	++	++			+
17	+				
18	++	+	+		
19	++				
20			+		+
21	++		+		+
22	+				
23	++				
24	+				
25	+				
26	++				
27	++				
28	++				

Figure 5.1 Detection of HCMV gB DNA in liver biopsies by ISH. Patient 1: A and B) HCMV gB DNA and C) negative control showing same section stained with no probe. Patient 2: D) and E) HCMV gB DNA and F) negative staining with HHV-6 U67 probe. Arrows indicate HCMV infected liver hepatocytes.

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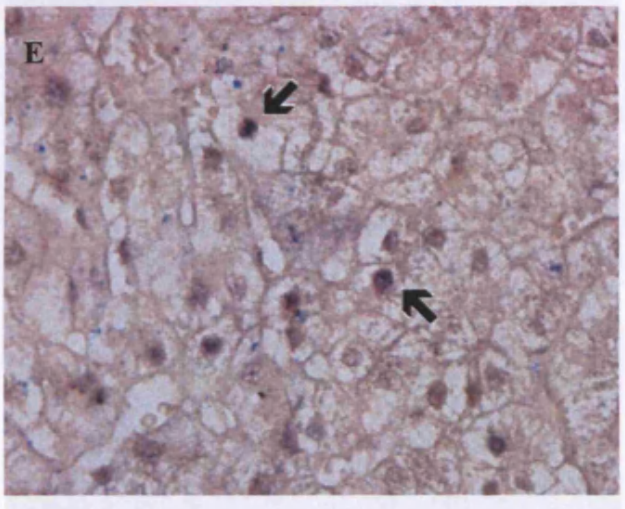
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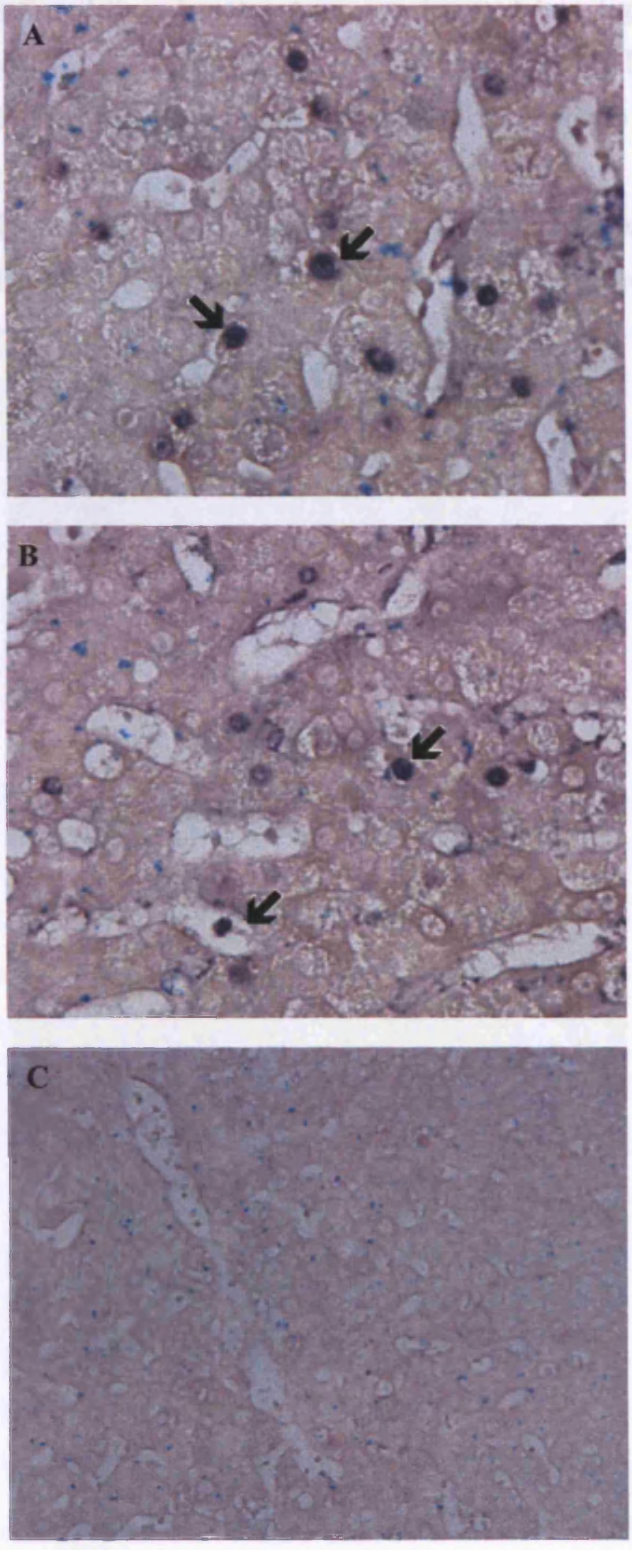
F

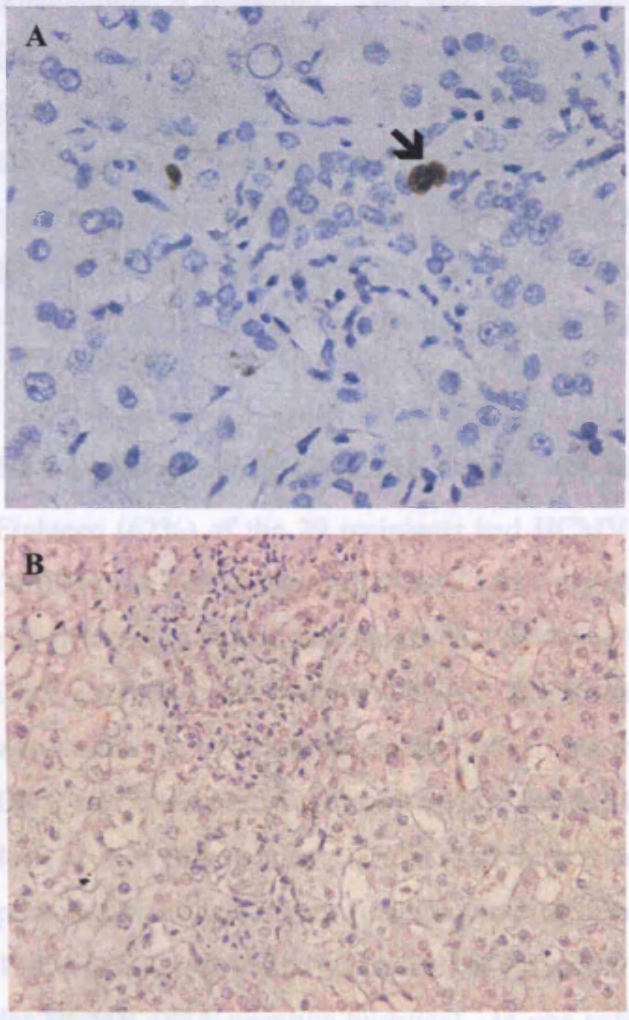
Figure 5.2 Liver biopsy showing comparison of hybridisation signals for HCMV gB and US28 DNA by ISH. A) HCMV gB DNA; B) HCMV US28 DNA; and C) negative staining with HHV-6 U67 probe. Arrows indicate positive hybridisation signals.



5.2.2 Detection of human β-herpesviruses proteins in liver biopsies by IHC

HCMV-specific late protein of 47-55 kDa was detected in only 2 of the 54 (4%) biopsies (one of which was independently shown to be positive for HCMV p52 protein by the Histopathology Diagnostic service at the Royal Free Hospital) from 2 of the 30 (7%) liver transplant patients. In these two liver biopsies, only a few occasional HCMV infected liver hepatocytes were detected (see figure 5.3). These 2 biopsies were not tested by ISH as there was insufficient material. Immunohistochemical staining for HHV-6 and HHV-7 late proteins on liver biopsies did not produce convincing results.

Figure 5.3 Liver biopsy from a patient showing HCMV late proteins by IHC. A) HCMV late proteins in liver hepatocytes (see arrows) and B) Isotype control.



5.2.3 HCMV serostatus and HCMV disease

Eighteen (60%) of the 30 recipients were HCMV seropositive pre-transplantation. Of those, 1 patient had a biopsy that was positive for HCMV late Ag as detected by IHC, but biopsy was insufficient for ISH. Ten (59%) of those 17 HCMV seropositive patients had HCMV DNA detected in their biopsy by ISH. Five of 9 (56%) seronegative patients had HCMV DNA detected in their biopsy by ISH suggesting virus transmission from the donor. However, two of these patients received a transplant from a seronegative donor. HCMV DNA was also detected in the blood by PCR 17 days post-transplant in one of these two patients.

Of the 30 recipients, 8 (27%) had biopsy-proven HCMV disease (1 gastric, 3 pneumonitis, 4 hepatitis). This was more common in the CMV D+R- serogroup: 5 (71%) of 7 developed HCMV disease versus 1 (14%) of 7 patients, and 1 (10%) of 10 patients in the D-R+ and D+R+ serogroups, respectively. Of those 8 recipients who developed HCMV disease, 5 (63%) had HCMV DNA detected in their biopsy by ISH (and in the blood by PCR). One patient had HCMV late Ags detected in the biopsy by IHC, but biopsy was insufficient for ISH. HCMV gB DNA was also detected in 12 (55%) out of 22 patients who did not develop HCMV disease ($p = 0.26$).

5.2.4 Correlation of HCMV ISH results with HCMV DNAemia

Eighteen (62%) of the 29 recipients had HCMV DNA detected in the blood by PCR. Of those, 10 (55%) also had HCMV DNA detected in their biopsy. HCMV DNA was also detected in at least one biopsy in 7 (64%) of 11 recipients who did not have HCMV DNA detected in the peripheral blood. In this study, consecutive liver biopsies ($n = 21$) were taken for 4 recipients during 120 d post-transplant. In all 4 recipients, HCMV DNA was detected in the biopsy by ISH before virus was detected in the blood by PCR. In 3 of 4 of these patients, HCMV DNA was detected in the biopsy early during the post-transplantation course (median time for detection of HCMV DNA in biopsies was 13 d (range 1 to 34) post-transplant, as compared to 51 d (range 3 to 83) post-transplant for HCMV DNA negative biopsies). In one patient HCMV DNA was not detected in biopsies taken early during the post-transplantation course (within 10 d post-transplantation), but in biopsies taken near the time of HCMV PCR positive blood (36 d post-transplantation). This patient had HCMV serostatus D+R+.

5.2.5 Correlation of HCMV ISH results with histological rejection

HCMV DNA was detected in 16/28 (57%) biopsies with no rejection, 8/16 (50%) biopsies with mild acute rejection, and 4/8 (50%) biopsies with moderate or severe acute histological rejection (p= .6065). Histological rejection was not more frequent in liver biopsies that were HCMV DNA positive by ISH.

5.2.6 Correlating HCMV ISH findings with the timing of post-transplant ALT peaks

The temporal relationships between detection of HCMV gB DNA in the biopsy by ISH, histological grading of the liver biopsies and timing post-transplant of "virus-associated ALT peaks" were examined. A peak was defined as a transient elevation in which the mean of the maximum value and the subsequent value was both at least 1.5 times higher than the previous lowest level and greater than 40 units/L. A "virus-associated ALT peak" was defined as one occurring within 7 days of PCR positivity for any virus (Griffiths *et al.*, 1999a).

Of the 52 liver biopsies (29 recipients), 24 (46%) biopsies (in 14 patients) were taken within 7 days of an ALT peak. Of those, 21 biopsies (12 patients) were taken during a human β-herpesvirus associated peak. Six biopsies (5 patients) were taken during HCMV associated peaks (1 showing mild rejection, 1 moderate rejection), 6 biopsies (5 patients) were taken during HHV-6 associated peaks (all showing mild-moderate rejection), 8 biopsies (6 patients) were taken during HHV-7 associated peaks (2 showing mild rejection, 1 severe rejection) and 1 biopsy (graded as moderate rejection) was taken during two peaks each associated with HHV-6 and HCMV.

Of the 24 biopsies taken within 7 days of an ALT peak, 14 (58%) biopsies were HCMV gB DNA positive. Of those, 12 biopsies were taken during a virus associated ALT peak. Four biopsies were taken during CMV associated peaks (1 showing moderate rejection), 5 biopsies during a HHV-6 associated peak (all showing mild-moderate rejection), 3 biopsies during a HHV-7 associated peak (1 mild rejection). Three out of the 28 (11%) HCMV gB DNA positive biopsies were taken with no ALT peak observed. Of those 5 HCMV gB DNA positive biopsies (5 patients) taken within 7 days

of a virus ALT peak showing no histological evidence of rejection, 4 were diagnosed cases of HCMV disease (1 hepatitis, 1 gastric, 2 pneumonitis).

5.3 Discussion and Conclusions

The results of this study show that HCMV DNA was frequently detected in biopsies from patients with liver allografts by ISH. Similar to other studies, HCMV DNA was predominantly detected in liver hepatocytes (Evans *et al.*, 1999; Arnold *et al.*, 1992) although other cell types including endothelial cells, Kupffer cells and inflammatory cells were found to contain HCMV DNA. CMV replication has been shown to be permissive in endothelial and epithelial cells (Ho *et al.*, 1984; Sinzger *et al.*, 1995).

In this study the biopsies containing HCMV DNA were not more likely to show graft rejection, therefore HCMV was not found to be a frequent trigger of rejection. Since the biopsies were taken during 120 d post-transplantation period, rejection in this case is likely to reflect that of an acute rather than of a chronic nature. There are very few studies investigating the role of HCMV in acute liver allograft rejection. One recent PCR-based study has shown that neither CMV viraemia or CMV disease adversely affects the incidence and grade of acute rejection episodes or the histological outcome of post-transplant HCV recurrence, during the first year after liver transplantation (Teixeira *et al.*, 2000). Most studies have focussed on the role of HCMV in the development of chronic rejection in liver transplant recipients, where the virus has been demonstrated in liver grafts with VBDS and in bile ducts and vascular structures of liver allografts associated with chronic rejection (Arnold *et al.*, 1992; Lautenschlager *et al.*, 1997a). These findings, however, have not been confirmed in other studies (Paya *et al.*, 1992; Wright, 1992).

HHV-6 infections complicating the post-transplantation period have also been reported in liver transplant patients (Herbein *et al.*, 1996; Singh *et al.*, 1997; Dockrell *et al.*, 1999). Several studies have suggested a role of HHV-6 in allograft rejection (Humar *et al.*, 2002; Lautenschlager *et al.*, 1998; 2000). In this study, HHV-6 or HHV-7 DNA was not detected in any of the liver biopsies using ISH of similar sensitivity, therefore these two viruses are unlikely to have a direct pathological role in graft rejection or dysfunction. This finding is in contrast to the situation in the blood where HCMV and HHV-6 were independently associated with biopsy proven graft rejection (Griffiths *et al.*, 1999a).

In this study, IHC was used to look for late HCMV proteins in liver allografts which is indicative of a productive infection. HCMV late proteins were only detected in two biopsies from two patients. The failure to detect HCMV late proteins shows that HCMV expression was at a low level, not detectable by IHC. HHV-6 or HHV-7 late proteins were also not detected in any of the liver biopsies. This finding is in contrast to studies conducted by Lautenschlager *et al.* (1998; 2000) where HHV-6 Ags were detected by immunohistochemical staining in liver biopsy specimens from liver transplant patients with graft rejection. In a study of 51 consecutive liver transplant patients, HHV-6 infection was diagnosed in 11 (22%) of patients during the first year after transplantation. Significant graft dysfunction with organ rejection was associated with the detection of HHV-6 antigenemia in 8 of the 11 patients, and importantly, HHV-6 Ags could also be detected by immunohistochemical staining in the liver biopsies of these 3 patients (Lautenschlager *et al.*, 2000). In our study, lack of detection of HHV-6 Ags in the liver biopsies could be due to technical differences in immunoshistochemical staining, and in the studies conducted by Lautenschlager's group frozen sections of liver biopsies were used, whereas our studies used paraffin-embedded liver biopsies which may affect the sensitivity of staining of the MAbs.

Studies have shown that the onset of HCMV infection, as indicated by the detection of HCMV in the blood by PCR is around 4 to 8 weeks after SOT (Kidd *et al.*, 2000; Griffiths *et al.*, 1999a). The exact time course of HCMV in liver allografts can only be determined in a prospective study testing protocol biopsies. In this study it was not feasible to test all protocolled liver biopsies during 120 day post-transplantation for this cohort of patients (biopsies were taken nearest the time of PCR positivity for any of human β-herpesviruses). Consecutive liver biopsies were only taken for four patients. In 3 of the 4 patients detection of HCMV DNA by ISH was early during the post-transplantation course (86% biopsies taken during 1-14 d post-transplantation were HCMV DNA positive), possibly suggesting virus transmission from the donor. Similar to the findings in the renal transplant patients (see chapter 4), a small population of liver transplant patients (24%) had HCMV DNA detected in at least one of their biopsy with no virus detected in the peripheral blood by PCR during 120 d post-transplantation. Again, this may reflect the role of local immune response in limiting virus reactivation to local sites, and thereby preventing widespread virus infection.

In this study, 56% seronegative patients had HCMV DNA detected in their biopsy by ISH suggesting virus transmission from the donor. However, two of these patients received a transplant from a seronegative donor. Our findings are similar to other studies where CMV DNA has been detected in tissue organs by ISH from seronegative individuals (Hendrix *et al.*, 1997; Kraat *et al.*, 1992). This may be explained by low levels of Ab that are not detectable by serological assays and hence resulting in a false negative CMV serostatus.

Because HCMV is a known cause of hepatitis confirmed by histological identification of CMV inclusion bodies, it was suggested that HHV-7 associated ALT peaks for which no rejection was observed may represent HHV-7 associated hepatitis (Griffiths *et al.*, 1999a). However, in this study, HHV-7 (or HHV-6) DNA was not detected by ISH in the liver biopsies taken during 7 days of an HHV-7 associated ALT peak for which no rejection was observed, therefore these two viruses are unlikely candidates for the cause of hepatitis. In this study, HCMV DNA was detected in 62% biopsies (from 5 patients) taken during 7 days of an ALT peak for which no rejection was observed. These may represent further cases of CMV hepatitis. The ISH method has been shown to be sensitive for detection of HCMV infected cells in liver biopsies. As mentioned previously the ISH method does not discriminate between active or latent infection. However, it is unlikely that the sensitivity of the assay is able to detect latent infection. Therefore, HCMV DNA in the liver allograft most likely represents low level active virus replication or abortive infection.

The findings in this study show that in contrast to the situation in the blood, the detection of HCMV and HHV-6 DNA in liver biopsies is not statistically associated with rejection. Although HHV-6 and HHV-7 are frequently detected in the blood by PCR of patients following transplantation, these two viruses were not detected in organs of liver (or renal) patients with graft dysfunction or rejection and are therefore unlikely to have direct pathogenic role in organ transplantation.

Chapter 6 – Development of Fluorescence *In Situ* Hybridisation using HHV-6 specific probes for the analysis of HHV-6 integration into human chromosomes.

6.1 Introduction

HHV-6 was first isolated from the peripheral blood of six patients with a variety of lymphoproliferative disorders and AIDS (Salahuddin *et al.*, 1986). HHV-6 is classified into two variants, HHV-6A and -6B, based on genetic and biological properties (Ablashi *et al.*, 1993), although no serological test can distinguish between Abs to either variant. HHV-6 is ubiquitous in the population with >90% seropositivity in adults. Infection with HHV-6 usually occurs within the first two years of life, and in this age group HHV-6 causes febrile illness including ES (Yamanishi *et al.*, 1988). As with other herpesviruses, HHV-6 persists in the host after primary infection by establishing a latent state possibly in monocytes and bone marrow progenitor cells (Kondo *et al.*, 1991; Luppi *et al.*, 1999), and chronic infection in salivary glands (Jarrett *et al.*, 1990). Reactivation of latent virus may occur under conditions of immunosuppression, such as in transplant recipients receiving immunotherapy, and thus resulting in various disease manifestations including encephalitis, pneumonitis, and bone marrow suppression (Carrigan *et al.*, 1991; Cone *et al.*, 1993; Drobyski *et al.*, 1993; 1994; Singh and Carrigan, 1996). In healthy immunocompetent individuals, HHV-6 can be detected in PBMCs by sensitive nested-PCR when sufficient quantities of DNA are tested (Clark *et al.*, 1996), suggestive of low levels of latent virus in the peripheral blood (Jarrett *et al.*, 1990; Clark *et al.*, 1996; Kidd *et al.*, 1996).

An alternative form of HHV-6 persistence characterised by very high viral loads in PBMC and integration of viral sequences into host cell chromosomes is in a minority of the population. This recognized phenomenon of chromosomal integration was first reported in three patients with abnormally high viral loads in PBMC that were detected by Southern blotting; 1 non-Hodgkin's lymphoma, 1 Hodgkin's disease, and 1 MS patient (Luppi *et al.*, 1993). Pulsed field gel electrophoresis (PFGE) analysis of PBMCs from these three patients showed fragments of molecular weight higher than 170 kb segment, indicating that the viral sequences are linked to high molecular weight cellular DNA. Further analysis of these three patients using FISH with HHV-6 specific probes showed in all three cases the presence of a specific hybridisation site, located on the short arm of chromosome 17 (Torelli *et al.*, 1995). Morris *et al.* (1999) suggested that the sites of integration were close to or in the telomeres of chromosome 17, in band p13.3 (17p13.3). In addition, the integrated viral sequences were typed as variant B in all three cases. Although the exact mechanism of HHV-6 integration is not yet know, it

may be of relevance that HHV-6 variants A and B have telomere-like repeat sequences present in the direct regions that flank the viral genome (Gompels and Macaulay, 1995; Isegawa *et al.*, 1999), a characteristic also found in Marek's Disease virus (an avian α-herpesvirus) which is known to integrate in to cellular chromosomes including telomere regions (Delecluse *et al.*, 1993).

Recently, integration has also been mapped to chromosome 22q13 and chromosome 1q44 in different individuals, and the possibility of chromosomal transmission of HHV-6 DNA has been reported (Daibata *et al.*, 1999; Tanaka-Taya *et al.*, 2004). A woman with Burkitts lymphoma, and her asymptomatic husband had HHV-6 DNA integrated at chromosome 22q13 and 1q44 respectively. Their daughter was found to have integrated virus at both these chromosomal sites suggesting that her viral genomes were inherited chromosomally from both parents (Daibata *et al.*, 1999). Recently, Tanaka-Taya *et al.* (2004) identified five unrelated individuals with persistent high HHV-6 DNA (4 variant A, 1 variant B), and an additional five members of their families with similar HHV-6 DNA levels in PBMC. In two of these families examined, a mother and son, and a father and daughter were all shown to have chromosomally integrated HHV-6. The concept of an 'endogenous herpesvirus' and its inheritance as a genetic element is completely novel. Alternatively, in the absence of inheritance, high viral loads found in PBMC could represent integration of viral sequences in a bone marrow progenitor cell which gives rise to a large number of cells/cell types in peripheral blood carrying copies of the viral genome.

In our own studies at the Royal Free, QC-PCR has been used to measure viral loads in a number of patient groups. In one study, HHV-6 loads in the PBMC of healthy individuals were investigated (Clark *et al.*, 1996). Using 1 μg of DNA in a nested qualitative PCR assay, the prevalence of HHV-6 was found to be 36% (9/25). Eight persons had viral loads ranging from 5-32 genome copies/μg DNA. The viral burden in the ninth individual (V1) was 1.2×10^6 HHV-6 genome copies/μg DNA which remained constant over a 10-month period (see table 6.1).

Sample Time (Weeks)	0	1	4	6	10	14	36	45
HHV-6 viral load (log10 genomes/μg PBMC DNA	6.08	5.90	6.04	5.85	6.08	6.00	6.15	6.18

Table 6.1 Consistently high viral loads in the peripheral blood of case V1 overtime by qualitative PCR (Clark *et al.*, 1996)

Furthermore, prospective studies carried out using the QC-PCR assay, have identified a number of transplant recipients with consistent abnormally high viral loads (approximately 10^7 viral genomes/ml blood) in all samples collected over time (Kidd *et al.*, 2000; Griffiths *et al.*, 1999a). Three such patients in 60 liver transplant patients (see figure 6.1), 1 of 52 renal patients and 1 of 71 bone marrow recipients have been identified. These are likely to be individuals with integrated virus as such viral loads are characteristic of this phenomenon. This would place the prevalence of integration at approximately 3% (Duncan Clark, pers. comm.). Tanaka-Taya *et al.* (2004) estimated the prevalence of integrated HHV-6 in a Japanese population, determined by consistently high viral loads in PBMC, at 0.21%.

It was hypothesized that if persons with integrated virus became stem cell donors, then genetic transmission of integrated virus to the recipient could be misinterpreted as active HHV-6 infection post-transplantation. By initiating pre-transplant screening of stem cell donors, a stem cell donor with a viral load of $8x10^6$ genomes/ml blood was identified. In this chapter, cell lines were established from V1 and from the stem cell donor by Dr Duncan Clark. The PBMCs from these individuals were immortalized by infection with EBV to give rise to B-lymphoblastoid cell lines (LCL). FISH was developed using HHV-6 specific probes for mapping the integration site of HHV-6 in these individuals.

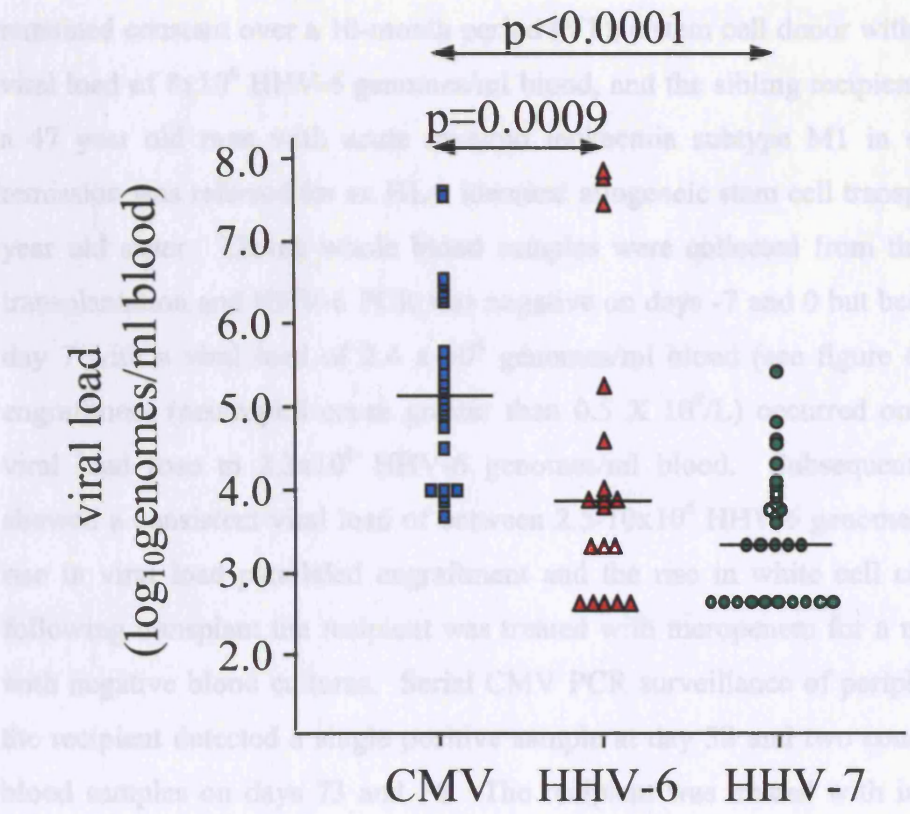


Figure 6.1 Maximum viral load of CMV, HHV-6 and HHV-7 in patients after liver transplantation (Griffiths *et al.*, 1999a). The horizontal bars indicate the median value for each of the β-herpesviruses.

6.2 Results

In this chapter, HHV-6 FISH was developed to map the site of viral integration in three individuals; a healthy individual with 1.2 x 10^6 HHV-6 genome/copies/μg DNA which remained constant over a 10-month period (V1), a stem cell donor with a pre-transplant viral load of 8x10^6 HHV-6 genomes/ml blood, and the sibling recipient. The recipient, a 47 year old man with acute myeloid leukaemia subtype M1 in second complete remission was referred for an HLA identical allogeneic stem cell transplant from his 37 year old sister. Eleven whole blood samples were collected from the recipient post-transplantation and HHV-6 PCR was negative on days -7 and 0 but became positive on day 7 with a viral load of 2.4 x 10^4 genomes/ml blood (see figure 6.2). Neutrophil engraftment (neutrophil count greater than 0.5 X 10^9/L) occurred on day 17 and the viral load rose to 2.3x10^6 HHV-6 genomes/ml blood. Subsequent weekly testing showed a consistent viral load of between 2.5-10x10^6 HHV-6 genomes/ml blood. The rise in viral load paralleled engraftment and the rise in white cell count. On day 7 following transplant the recipient was treated with meropenem for a neutropenic fever with negative blood cultures. Serial CMV PCR surveillance of peripheral blood from the recipient detected a single positive sample at day 38 and two consecutive positive blood samples on days 73 and 76. The recipient was treated with intravenous GCV (10mg/kg per day) which was discontinued after 5 days when CMV PCR became negative. He was discharged from this transplant centre five months following transplantation but relapsed 3 months later.

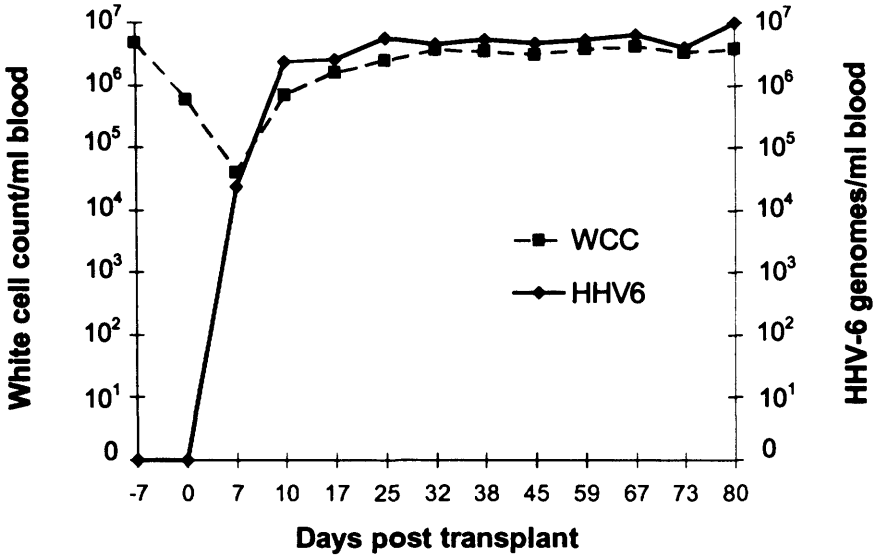


Figure 6.2 HHV-6 viral load in a stem cell transplant recipient who received a transplant from a sibling donor with viral load of $8x10^6$ HHV-6 genomes/ml blood.

Metaphase chromosomes were successfully generated from the LCLs of the healthy individual (V1) and the stem cell donor but not from the recipient. Cultures of the recipient cell line grew very slowly and a seeding density of $1x10^6$/ml could not be achieved. Therefore, FISH was performed only for V1 and the stem cell donor. The development of FISH using direct labelling of HHV-6 plasmid pZVH14 (see section 2.8.1) did not produce convincing results. No specific hybridisation signals could be visualized for both cell lines. For signal amplification, the HHV-6 pZVH14 probe was also indirectly labelled with biotin-dUTP, and secondary detection was carried out using fluorescein conjugated avidin (see section 2.8.2). Hybridisation was also carried out at 24 and 48 hr, and a blocking step was incorporated to improve non-specific hybridisation. Again, no specific hybridisation signals were observed for both cell lines. FISH was further developed using a cocktail of eight plasmids containing between 9 and 16 kb inserts of the HHV-6 genome (nonoverlapping) (see figure 2.4) as probes (7 plasmids were kindly provided by Dr Scott Schmid, Centres for Disease Control, Atlanta (Lindquester *et al.*, 1996), and plasmid pZHV14 (kindly provided by Professor Ruth Jarrett, University of Glasgow, UK). These plasmids were directly

labelled as described in section 2.8.3. Using a cocktail of HHV-6 specific plasmids as probes for FISH, the viral genome was found to be integrated close to the end of the short arm of chromosome 17 for the stem cell donor. For V1, HHV-6 viral genome was integrated on the short arm of chromosome 11. Figure 6.3 show specific symmetrical doublet signals on both chromatids of a single homolog of chromosome 17 for the stem cell donor and chromosome 11 for V1. This part of the FISH was carried out in collaboration with Drs Nacheva, Brazma (Haematology) and Leong (Virology).

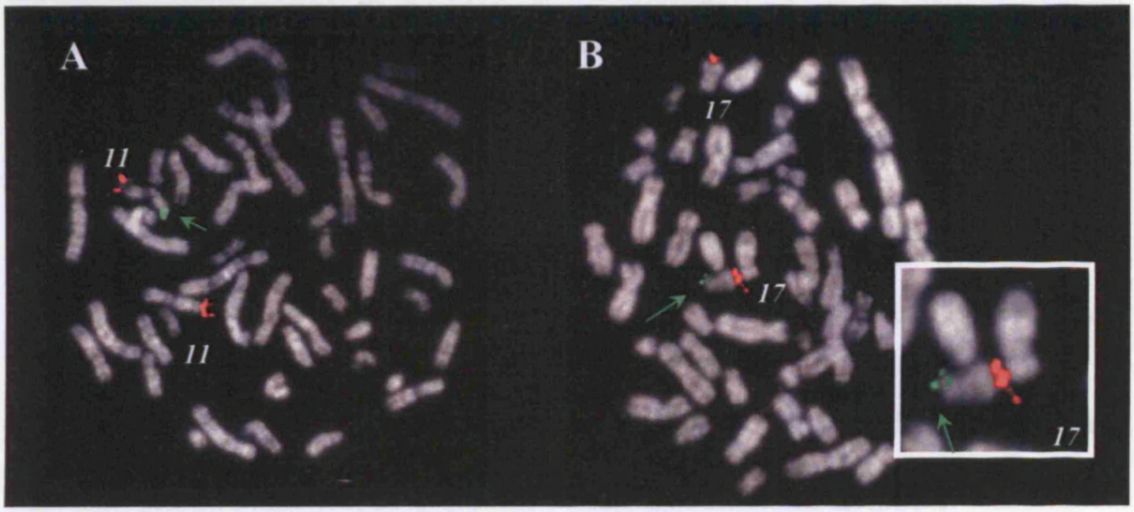


Figure 6.3 FISH analysis using fluorescently labelled HHV-6 specific probes showing integration of HHV-6 sequences on A) chromosome 11p in V1-LCL and B) chromosome 17p in LCL of the stem cell donor (pictures provided by D.Clark).

Separate analysis using PCR primers to each of the predicted ORFs in the unique region of the viral genome conducted by Edward Tsao and Hoe Nam Leong in our department has shown that the complete genome is present in both cell lines and is variant B in V1 and variant A in the stem cell donor.

6.3 Discussion and Conclusions

Our FISH results identified integration of HHV-6 sequences in chromosome 11p and 17p for V1-LCL and the donor LCL, respectively. Subsequent FISH using HHV-6 probe combined with Spectrum Orange labelled telomere probe specific for either the long arm of chromosome 11 or 17 (done in collaboration with Dr Nacheva) have confirmed the specific integration site of HHV-6 sequences on chromosome 11p15.5 and 17p13.3 for V1-LCL and the LCL of the stem cell donor, respectively. Similar analysis on a bone marrow preparation from the recipient 108 days pre-transplant showed no evidence of integrated HHV-6. FISH analysis on PBMC and bone marrow samples on days 31 and 88 post-transplant based on XX and XY chromosome status showed successful engraftment. In a bone marrow sample taken on day 88 post-transplantation from the recipient, HHV-6 sequences have been identified on chromosome 17p13.3 (D.Clark, pers. comm.).

Results in this chapter, and subsequent FISH analysis of the donor LCL and recipient bone marrow post-transplantation have confirmed integrated sequences present on chromosome 17p, a site where integration has previously been identified (Luppi *et al.*, 1993; Torelli *et al.*, 1995; Morris *et al.*, 1999). HHV-6 integration has also been mapped to chromosome 22q13 and 1q44 (Daibata *et al.*, 1999). In this study, the HHV-6 integration site for VI-LCL was located in the short arm of chromosome 11. This has not been previously reported elsewhere, and represents another integration site for the virus.

At present the underlying molecular mechanisms which control integration of HHV-6 to chromosomal sites are unknown. All reported integration sites are in the most telomeric regions. The HHV-6 viral genome contains a region that is homologous to the human telomeric sequence (Gompels and Macaulay, 1995). Thus viral integration into the human telomeric region may occur by a targeted recombination event (Tanaka-Taya *et al.*, 2004). Further fine mapping of the site of integration for these individuals will be required to determine whether integration is close to or within telomeric regions of the viral genome. HHV-6 is unique amongst the human herpesviruses by encoding an ORF (U94) homologous to the human adeno-associated virus type-2 (AAV-2) *rep* gene products Rep68 and Rep78 (Thomson *et al.*, 1991). The AAV-2 *rep* gene plays an essential role in viral DNA replication and has been shown to regulate gene expression.

HHV-6 U94 has been shown to complement rep in function in replication defective AAV-2 (Thomson *et al.*, 1994). The AAV-2 *rep* gene products Rep68 and Rep78 are also thought to play an important role in AAV-2 integration and can target DNA sequences to a specific locus in the human genome (Surosky *et al.*, 1997). Since HHV-6 U94 shows homology to both these AAV proteins it may be that it functions in a similar manner by binding to recognition sequences present in particular chromosomes.

We have demonstrated that a stem cell recipient can become positive for HHV-6 DNA by PCR when engraftment takes place from a donor with integrated HHV-6. The pattern of HHV-6 viral loads for this recipient (see figure 6.2) could easily be misinterpreted as active infection prompting administration of potentially toxic antiviral drugs such as GCV. From a management perspective, pre-transplant screening of donors would therefore be useful in identifying those with integrated virus and therefore prevent misdiagnosis of active infection post-transplantation. To date there have been no controlled trials of antiviral therapy against HHV-6 infection, but a number of studies have shown the ability of GCV and PFA to suppress HHV-6 replication *in vitro* (Takahashi *et al.*, 1997b).

Although the clinical significance of chromosomally integrated HHV-6 is still unclear, integrated virus is a potential confounder in studies investigating HHV-6 disease associations and also in the medical management of infection. Previous unproven associations between HHV-6 and disease, may have reflected the unrecognized detection of integrated virus and interpreted as a high viral load. For example, in a study by Challoner *et al.* (1995) representational difference analysis (RDA) was used to look for novel DNA sequences in the brains of MS patients compared to control tissue (PBMCs from healthy persons). An HHV-6 sequence was amplified from the brain of one MS case (MS-1). Limiting dilution analysis was also performed to estimate the viral load in MS and control brain samples. MS-1 produced a positive PCR up to a dilution of 1/262000, and a second MS brain was positive to a dilution of 1/65000. In contrast a maximum of 1/16 dilution was amplified from the other 47 MS and 51 control brains. The CSF of MS-1 was also HHV-6 DNA positive by PCR. Therefore, it is likely that this individual with abnormally high HHV-6 load in the brain had integrated HHV-6, possibly inherited, and the virus is unlikely to be associated with disease pathogenesis in this case.

In summary, this chapter describes the development of HHV-6 FISH using a cocktail of HHV-6 probes that can now be utilised to examine sites of virus integration. We propose that all stem cell donors should be screened for integrated HHV-6 and suggest that integration should be excluded in all reports associating this virus with novel disease associations.

Chapter 7 - General Discussion and Conclusions

7.1 General Discussion and Conclusions

HCMV is the most important post-transplant infection. It causes overt end-organ disease such as hepatitis, pneumonitis, and gastrointestinal ulceration, (Griffiths and Emery, 1997). These are collectively termed the "direct effects" of CMV because the virus can be seen directly in biopsies of the infected organ using classical histopathological techniques to visualise "owl's eye inclusions" (Smith, 1959; Macasaet *et al.*, 1975). In addition, HCMV is associated with a variety of other medical conditions (graft rejection, accelerated atherosclerosis and an immunosuppressive syndrome (Rubin, 1989), collectively termed "indirect effects" because the virus is not seen histopathologically in affected organs.

The involvement of CMV infection in transplant rejection was first proposed by Richard Simmons and coworkers in 1970. Since then, several studies based upon clinical observations in individual patients, statistical association with CMV disease and/or CMV infection in cohorts of patients as well as the results of clinical trials of antiviral agents have provided further evidence for a causal role of CMV in allograft rejection (Grattan *et al.*, 1989; Loebe *et al.*, 1990; Bando *et al.*, 1995; von Willebrand *et al.*, 1986; Fietze *et al.*, 1994; Reinke *et al.*, 1994; Valantine *et al.*, 1999; Rubin *et al.*, 2000). Specifically, the trial of Lowance *et al.* (1999) randomised 208 HCMV seronegative patients to receive prophylaxis with VACV or placebo for ninety days after transplant of a seropositive kidney, reported that the incidence of biopsy-proven acute graft rejection was decreased by 50% among those receiving VACV. This finding, together with supporting evidence from animal models of allotransplantation in the presence of animal CMVs with/without GCV therapy, strongly indicated that CMV was an unrecognized major contributor to acute graft rejections (Lemstrom *et al.*, 1995; 1997).

However, evidence against a causal role for HCMV in triggering allograft rejection also exists. Several clinical cohort studies have not reported a statistical association with HCMV (Dickenmann *et al.*, 2001; Sherlock *et al.*, 1991; Teixeira *et al.*, 2000; Boyce *et al.*, 1988). In the Lowance study, a parallel group of 408 renal transplant patients who were HCMV seropositive pre-transplant also did not demonstrate a beneficial effect of VACV prophylaxis on the incidence of biopsy-proven graft rejection. Immunosuppression associated with transplantation may also enhance the replication of

other β-herpesviruses, namely HHV- 6, and/or HHV-7. These two viruses have also been associated with graft rejection and with HCMV end organ disease (Griffiths *et al.*, 1999a; Kidd *et al.*, 2000; Osman *et al.*, 1996; Desjardin *et al.*, 1998; Ratnamohan *et al.*, 1998). Since HHV-6 and to a lesser extent HHV-7, are susceptible *in vitro* to drugs which inhibit HCMV including GCV (De Clercq *et al.*, 2001) it remains possible that the beneficial effects of antiviral prophylaxis on acute graft rejections may be operating through inhibition of these newer β-herpesviruses and/or inhibition of HCMV.

To address this complex situation prospective studies were conducted in our centre where QC-PCR assays were applied to quantitatively measure the viral load of each of these three β-herpesviruses in renal (Kidd *et al*, 2000) and liver (Griffiths *et al.*, 1999a) transplant patients. In the study by Kidd *et al.* (2000), HCMV was found to be the most commonly detected virus post-transplant (58% of patients) followed by HHV-7 (46%) and HHV-6 (23%). The median maximum viral loads for HCMV was also significantly higher than those for HHV-6 ($p=0.01$) and HHV-7 ($P>0.0001$), and a trend for HHV-7 viral load to be greater than HHV-6 ($p=0.08$). It was revealed that in those patients with graft rejection, HHV-7 was associated with more episodes of rejection ($p=0.02$). There was also a significant increase in HCMV disease occurring in patients with HCMV and HHV-7 coinfection compared to those with HCMV infection only ($p=0.04$). Therefore, HHV-7 should be further investigated as a possible co-factor in the development of HCMV disease in renal transplant patients and may potentially exacerbate graft rejection. Similarly, in the study by Griffiths *et al.* (1999a), infections with HHV-6 and HHV-7 were also found to be common after transplantation. The median peak virus load for HCMV was again significantly greater than that for HHV-6 and HHV-7. HCMV and HHV-6, but not HHV-7 were also found to be independently associated with biopsy proven graft rejection. It was therefore concluded that HHV-6 may be a previously unrecognized pathogen in this patient group (Griffiths *et al.*, 1999a). However, to fully understand the pathogenic role of these viruses in organ transplantation, detection of these viruses must be made at the tissue level. Traditionally, the diagnosis of HCMV direct end organ disease has relied on classical histopathological detection of "owl's eye inclusions" in the biopsies of the affected organ. Although this method was specific for HCMV (inclusion bodies were not induced by HHV-6 or HHV-7 *in vivo*), it was found to be insensitive (Mattes *et al.*, 2000).

One of the main aims of this thesis was therefore to develop specific and more sensitive *in situ* techniques including ISH and IHC for the detection of the human β-herpesviruses in organ biopsies to further investigate the incidence and pathogenesis of these viruses in transplant recipients. Chapter 3 describes the development of ISH using DIG labelled riboprobes specific for each of the human β-herpesviruses for the detection of virus DNA in formalin-fixed paraffin-embedded culture infected cells. Furthermore, HCMV ISH was optimised for the detection of HCMV infected cells in biopsy material. A DIG labelled riboprobe directed against the HCMV IE-1 gene transcript for RNA ISH was also constructed to further investigate HCMV gene expression in renal biopsies. A riboprobe specific for a β-globin sequence was used to determine the level of sensitivity of the ISH technique. Detection of DNA was found not to approach single-copy levels. However, HHV-6 DNA was detected by ISH with a HHV-7 U67 riboprobe in an EBV-transformed cell line with an estimated 12copies of integrated HHV-6 genome (VI-LCL). IHC was also developed using MAbs specific for each of the human β-herpesvirus proteins in formalin-fixed paraffin-embedded culture infected cells. These assays were shown to be sensitive and specific for the respective virus. For IHC, a number of MAbs were used with optimal staining obtained with MAbs to HCMV late protein, HHV-6 gB and HHV-7 pp85.

The *in situ* techniques were then used for the detection of these viruses directly in renal biopsies (Chapter 4) taken from the patients in the original cohort who required biopsy to investigate the cause of graft dysfunction (Kidd *et al.*, 2000), and also in liver biopsies (Chapter 5) taken from the patients in the original cohort where protocolled liver biopsies were obtained at scheduled times after transplantation, and whenever liver dysfunction occurred (Griffiths *et al.*, 1999a). Since it was not feasible to test all liver biopsies, biopsies were selected from patients taken nearest the time of PCR positivity in the blood for any of the human β-herpesviruses.

Using ISH, HCMV DNA was detected in a significant proportion of biopsies (approximately 50%) from patients with renal and liver allografts, with detection being widespread especially in the renal allografts. The renal biopsies containing HCMV DNA were however, not more likely to show graft rejection so the hypothesis that HCMV is a frequent trigger of rejection was not supported. Instead, it is proposed that HCMV infection of the graft causes post transplant graft dysfunction which mimics

rejection clinically and which can be prevented by antiviral prophylaxis such as that used by Lowance *et al.* (1999). Similarly, HCMV DNA was not more likely to be detected in liver biopsies showing graft rejection, therefore HCMV was also found not to be statistically associated with rejection in this patient group.

In the renal allografts, HCMV IE-1 gene transcripts were also not detected by RNA ISH, and this may be due to the quality of the biopsies as β-actin transcripts were only detected (and in low abundance) in some renal allografts suggesting RNA degradation. Therefore, although HCMV DNA was detected in the renal and liver allografts, the type of HCMV infection could not be established by ISH. The failure to detect HCMV Ags shows that HCMV expression was at a low level and explains why this phenomenon has not been described previously by histopathologists using IHC or examination for owl's eye inclusions which are known to be insensitive (Mattes *et al.*, 2000; Smith *et al.*, 1975). However, the sensitivity of the ISH technique is unlikely to detect latent infection, so the HCMV DNA in the biopsies most likely represents low level virus replication or abortive infection.

In renal (and cardiac) transplantation, high levels of AECA have been demonstrated in transplant recipients who had HCMV infections (Toyoda *et al.*, 1997; 1999). These Abs have been proposed to play an important role in the development of Ab-mediated rejection (Yard *et al.*, 1993; Fredrich *et al.*, 1999). The deposition of C4d in the PTC of the renal graft was used to investigate the incidence of Ab mediated injury in the renal transplant patients. Approximately 50% of biopsies showed C4d staining, this finding is similar to that of other studies (Lederer *et al.*, 2001; Feucht *et al.*, 1993). The detection of HCMV DNA in the renal biopsies by ISH was not associated with Ab mediated rejection as determined by C4d deposition. This finding is in line with a recent study by Nickeleit *et al.* (2002), where clinical evidence of infections within 2 weeks before a renal allograft biopsy (HCMV, VZV, sepsis, bacterial infections) were not significantly associated with C4d deposition (Nickeleit *et al.*, 2002). However, a recent study by Aiello *et al.* (2004), showed the presence of PTC C4d deposition in the graft of some renal transplant patients undergoing early acute rejection and who experienced viral infections (CMV or EBV) within the first year following transplantation.

HHV-6 or HHV-7 were not detected in organs of liver or renal transplant patients by ISH or IHC. This finding is in contrast to the situation in the blood where HHV-6 and HHV-7 DNA were commonly detected in the blood by PCR (Griffiths *et al.*, 1999a; Kidd *et al.*, 2000). HHV-7 was also associated with increased episodes of rejection in the renal patients (Kidd *et al.*, 2000), and HCMV and HHV-6 were independently associated with biopsy proven graft rejection in the liver patients (Griffiths *et al.*, 1999a). The role of these β–herpesviruses in graft dysfunction or rejection is therefore also not supported. Although the findings in this thesis do not support a role of the newer β-herpesviruses as major pathogens in solid organ transplant recipients, these viruses were however, only examined in the liver and kidney which may not necessarily be the site of HHV-6/7 disease. Several studies have shown that HHV-6 may be associated with other disease associations such as encephalitis and bone marrow suppression (Drobyski *et al.*, 1994; Carrigan and Knox, 1994; Imbert-Marcille *et al.*, 2000), therefore HHV-6 may still be an important pathogen post-transplant.

In the renal transplant population there was a trend towards the presence of HCMV DNA in the graft as detected by ISH and the long-term outcome among the renal transplant recipients although this did not achieve statistical significance. This study was however, limited to low patient numbers and future work could involve extending this study to incorporate a larger number of renal transplant patients. In the liver transplant patients it was not feasible to test all protocolled liver biopsies during 120 day post-transplantation for this cohort of patients (biopsies were taken nearest the time of PCR positivity for any of human β-herpesviruses). Consecutive liver biopsies were only tested for four patients, therefore future work could involve extending this to a larger number of patients which would help determine the exact time course of HCMV in liver allografts. In our centre, formalin-fixation followed by tissue processing for paraffin-embedding is the routine method for preservation of tissue biopsy for microscopic analysis. Future work could also involve testing frozen liver and renal biopsies from transplant patients to compare our *in situ* results, and also those of the findings by Lautenschlager's group, where HHV-6 Ags were detected by IHC in frozen liver sections.

In conclusion, the finding that HCMV DNA is frequently detected in biopsies by ISH shows that rejection and infection frequently co-exist. A common clinical conundrum in patients who present with fever and graft dysfunction is the differential diagnosis of rejection *or* infection. The *in situ* hybridisation method for HCMV has been demonstrated to be a sensitive method for the detection of HCMV infected cells in liver and renal biopsies. It could be used to further test biopsies collected during clinical trials allowing patients to be randomised to receive GCV or placebo; the results would then determine whether patients with graft dysfunction should be given anti HCMV therapy in addition to treatment for their graft rejection.

Although the role of HHV-6 in graft dysfunction or rejection was not shown, a number of transplant recipients were identified with consistent abnormally high viral loads (approximately 10^7 viral genomes/ml blood) in all samples collected over time (Kidd *et al.*, 2000; Griffiths *et al.*, 1999a). In another study, where 1 μg of DNA was used in a nested qualitative PCR assay to measure HHV-6 loads in the PBMC of healthy individuals, the prevalence of HHV-6 was found to be 36% (9/25) (Clark *et al.*, 1996). Eight persons were found to have viral loads ranging from 5-32 genome copies/μg DNA. The viral burden in the ninth individual (V1) was 1.2 x 10^6 HHV-6 genome copies/μg DNA which remained constant over a 10-month period. These are likely to be individuals with integrated virus as such viral loads are characteristic of this phenomenon. This would place the prevalence of integration at approximately 3% (Duncan Clark, pers. comm.). Tanaka-Taya *et al.* (2004) estimated the prevalence of integrated HHV-6, determined by consistently high viral loads in PBMC, at 0.21% in the Japanese population.

It was hypothesized that if persons with integrated virus became stem cell donors, then genetic transmission of integrated virus to the recipient could be misinterpreted as active HHV-6 infection post-transplantation. By initiating pre-transplant screening of stem cell donors, a stem cell donor with a viral load of $8x10^6$ genomes/ml blood was identified. Chapter 6 describes the development of FISH using a cocktail of HHV-6 specific probes for mapping the integration site of V1-LCL and the stem cell donor. Attempts to generate an LCL from the stem cell recipient were unsuccessful as B cell counts were very low in peripheral blood. Our FISH results identified integration of HHV-6 sequences in chromosome 11p and 17p for V1-LCL and the donor LCL,

respectively. Subsequent FISH analyses (conducted by Dr Elisabeth Nacheva, Dr Diana Brazma and Dr Hoe Nam Leong) confirmed the specific integration site of HHV-6 sequences on chromosome 11p15.5 and 17p13.3 for V1-LCL and the LCL of the stem cell donor, respectively. Similar analysis on a bone marrow preparation from the recipient 108 days pre-transplant showed no evidence of integrated HHV-6. FISH analysis on PBMC and bone marrow samples on days 31 and 88 post-transplant based on XX and XY chromosome status showed successful engraftment. In a bone marrow sample taken on day 88 post-transplantation from the recipient, HHV-6 sequences have been identified on chromosome 17p13.3 (Duncan Clark, pers. comm.). We have demonstrated that a stem cell recipient can become positive for HHV-6 DNA by PCR when engraftment takes place from a donor with integrated HHV-6. We propose that pre-transplant screening of donors can be useful in identifying those with integrated virus and therefore prevent misdiagnosis of active infection post-transplantation

Whilst the clinical significance of chromosomally integrated HHV-6 is still unclear, integrated virus is a potential confounder in studies investigating HHV-6 disease associations and also in the medical management of infection. Previous unproven associations between HHV-6 and disease, may have reflected the unrecognized detection of integrated virus and interpreted as a high viral load. Therefore, it is recommended that integration should be excluded in all reports associating this virus with novel disease associations.

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