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**Virological and Immunological studies of
human cytomegalovirus in transplant
patients**

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A thesis submitted to the University of London in the
Faculty of Clinical Science for the degree of Doctor of Philosophy

September 2004

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Finally, I would like to pay tribute to all the patients who entered the studies, without their participation this work would not have been possible.

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Summary

Infection with human cytomegalovirus (HCMV), a member of the *Betaherpesvirinae* sub-family is widespread. HCMV is a major pathogen in the fetus, transplant patients (solid and bone marrow) and patients infected with the human immunodeficiency virus (HIV).

My first study analyzed the presence of HCMV, human Herpes Virus 6 (HHV 6) or human Herpes Virus 7 (HHV 7) by quantitative competitive polymerase chain reaction (QCPCR) in multiple organs collected from acquired immunodeficiency disease syndrome (AIDS) patients autopsy. The results showed that the presence of owl's eye inclusion bodies is highly specific for HCMV, but has a low sensitivity.

My second study investigated T-cell mediated immunity as factor in controlling HCMV viral replication. Post renal transplantation, HCMV specific CD8 T-cells were enumerated with 'tetrameric complexes' and the functional capacity of these cells was assessed by an interferon gamma (IFN- γ) secretion assay. The results showed a higher frequency of specific CD8 T-cells in viremic patients and confirmed a functional deficiency in HCMV specific CD8 T-cells, present before during and after HCMV viremia.

My third study analysed the data from a randomized controlled clinical trial, comparing intravenous (iv) ganciclovir (GCV) versus a combination of iv GCV plus foscarnet (PFA), each at half dose was performed, in liver, renal and bone marrow transplant (BMT) patients. The study showed no advantage for combination therapy compared to the monotherapy with iv GCV. Moreover, there was a trend in favour to GCV. All drug related toxicity was observed in the combination arm.

Valganciclovir (VGCV) is an oral prodrug of GCV. An uncontrolled study compared the virological response to either iv GCV or oral valganciclovir (VGCV) in solid organ transplant (SOT) patients and showed that the virological response was identical for this preparation of GCV.

In the the final part, I explored factors which predict the length of antiviral therapy required to become HCMV DNAemia negative. Univariable and multivariable regression models identified maximum virus load and baseline virus load (virus load at the beginning of antiviral therapy) as the strongest predictors for the length of antiviral therapy in the HCMV immune experienced patient group.

Overall, the results in this thesis address the complex interactions of HCMV with the host immune response and provide evaluations of methods used to measure these directly in patients.

Contents

Bibliography	1
Acknowledgments	1
Summary	2
Abbreviations	13
1 General Introduction	16
1.1 Human Cytomegalovirus	17
1.1.1 Introduction	17
1.1.2 Identification and Classification	17
1.1.3 Virus structure	18
1.1.4 Important proteins of HCMV and location within the HCMV genome	22
1.1.5 Capsid Proteins	22
1.1.6 Tegument Proteins	22
1.1.7 Envelope Proteins	23
1.1.8 Transactivators	24
1.1.9 Genes important for antiviral therapy/resistance	24
1.1.10 Gene homologs of chemokine receptors or chemokines	25
1.1.11 HCMV replication	25
1.1.12 Herpesvirus cascade	26
1.1.13 Virion assembly, maturation and egress	29
1.1.14 Growth in-vivo and in-vitro	29
1.2 Epidemiology	30
1.3 Transmission of HCMV	30
1.3.1 Intrauterine infection	30
1.3.2 Perinatal infection	31
1.3.3 Postnatal infection	31
1.3.4 Blood transfusion	31
1.3.5 Transmission during solid organ transplantation	31
1.3.6 After bone marrow transplantation	32

1.4	Pathogenesis and Pathology	32
1.4.1	Tissue Tropism	33
1.4.2	Factors correlating with disease production	33
1.4.3	Direct effects of HCMV infection	35
1.4.4	Indirect effects of HCMV infection	35
1.4.5	Neonates	38
1.4.6	The immunocompromised patient	39
1.5	Treatment of HCMV infection or disease	41
1.5.1	Activity of drugs in-vitro	41
1.5.2	Acyclovir	42
1.5.3	Valaciclovir	45
1.5.4	Ganciclovir	45
1.5.5	Valganciclovir	47
1.5.6	Foscarnet	47
1.5.7	Cidofovir	48
1.5.8	Fomivirsen	50
1.5.9	Evidence-base for treatment	50
1.6	Detection of systemic HCMV infection	54
1.6.1	Cell culture	54
1.6.2	Detection of HCMV in organs or tissue biopsies	54
1.6.3	Antigenaemia	55
1.6.4	Molecular amplification methods	55
1.7	Vaccines	55
1.7.1	Live attenuated vaccine	56
1.7.2	Soluble HCMV gB based vaccine	57
1.7.3	Canarypox based pp65 vaccine	57
1.7.4	Peptide based HCMV vaccines	58
1.7.5	HCMV DNA vaccine	58
1.8	Immune response against HCMV	58
1.8.1	Humoral immunity	58
1.8.2	Cellular immune response	59
1.8.3	Immune evasion	61
1.9	Aims of the thesis	63
2	Materials and Methods	65
2.1	Isolation of peripheral blood mononuclear cells from whole blood	66
2.1.1	Counting cells and assessing cell viability	66
2.1.2	Cryopreservation of peripheral blood mononuclear cells	67

2.2	Thawing of Lymphocytes	67
2.3	Construction of HCMV HLA peptide tetrameric complexes	67
2.3.1	Peptides	67
2.3.2	Recombinant E.coli	68
2.3.3	Expression of β_2 -microglobulin and HLA heavy chains in E. coli . . .	68
2.3.4	Inclusion body purification	69
2.3.5	Refolding of monomeric HLA-peptide synthetic molecules	69
2.3.6	Biotinylation of HLA-peptide monomers	69
2.3.7	FPLC purification of the refolded biotinylated monomers	70
2.3.8	Ion exchange purification of the selected FPLC fraction	70
2.3.9	Protein complex conformation EIA	70
2.3.10	EIA to test for biotinylation	71
2.3.11	Concentration of correctly folded and biotinylated protein fractions	71
2.3.12	Tetramer formation with Streptavidin	71
2.4	Immunostaining with HCMV pp65 tetrameric complexes	74
2.5	Immunostaining of PBMCs for lymphocyte surface markers	74
2.6	Aquisition of data and flow cytometric analysis	74
2.6.1	Gating strategy and for enumerating HCMV pp65 specific CD8 T-cells	75
2.7	CD8 ELISpot assay	77
2.7.1	Microtiterplate coating	77
2.7.2	Preparation of antigens and cells	77
2.7.3	Developing ELISpot plate	77
2.7.4	Analysis of ELISpot plate	78
2.8	Extraction of viral DNA from whole blood or paraffin embedded tissue samples	80
2.8.1	Extraction of DNA from whole blood	80
2.8.2	Extraction of DNA from paraffin embedded tissue samples	80
2.9	Detection of HCMV by PCR	81
2.9.1	Qualitative HCMV PCR	81
2.9.2	Quantitative competitive HCMV PCR (QC-PCR)	82
2.9.3	Quantitative TaqMan HCMV PCR	82
2.9.4	Quantitative competitive HHV-6 and HHV-7 PCR	86
2.10	Mathematical models for HCMV replication kinetics	87
2.10.1	Calculation of replication and decline rate (ρ and κ)	88
2.10.2	Calculating the replication rate on serial virus load data	88
2.10.3	Calculation of decline rate (κ)	88
2.10.4	Calculating the decline rate on serial virus load data	89

2.11	Statistical methods	92
2.11.1	Comparison of continuous variables	92
2.11.2	Independence of categorical data	93
2.11.3	Linear regression analysis	93
2.11.4	Univariable logistic regression	94
2.11.5	Multivariable logistic regression model	94
2.11.6	Survival analysis	95
2.11.7	Receiver operator curve	95
3	Detection of owl's eye inclusions by histology	96
3.1	Introduction	97
3.2	Materials and Methods	98
3.2.1	Clinical samples	98
3.2.2	Histopathological examination	98
3.2.3	Extraction of DNA from paraffin wax embedded tissue	98
3.2.4	Methods for PCR and QCPCR for HCMV, HHV-6 and HHV-7	99
3.3	Results	100
3.3.1	Relationship between viral load and the presence of owl's eye inclusion bodies	100
3.3.2	Quantitative relation between HCMV and owl's eye inclusion in different organs	103
3.4	Discussion	107
4	Natural History Study of CD8+ T-Cell Mediated Immunity against HCMV in Renal Transplant Patients	108
4.1	Introduction	109
4.2	Materials and Methods	110
4.2.1	Study population and study design	110
4.2.2	HCMV surveillance	110
4.2.3	Immunosuppression	110
4.2.4	Antiviral prophylaxis	111
4.2.5	Pre-emptive antiviral treatment	111
4.2.6	Immunological monitoring	111
4.3	Results	113
4.3.1	Demographic data of study cohort	113
4.3.2	Age and control of HCMV replication	113
4.3.3	Virological Markers of Study Cohort	113
4.3.4	Quantification of HCMV pp65 specific CD8+ T-cells with tetrameric complexes	114

4.3.5	Enumeration of functional HCMV specific CD8+ T-cells in HCMV seropositive patients	115
4.3.6	Functional capacity of ppUL83 specific CD8+ T-cells and failure to control HCMV replication	115
4.3.7	Factors associated with high level HCMV replication post renal transplantation	116
4.4	Discussion	131
5	A randomized, controlled trial comparing ganciclovir or ganciclovir plus foscarnet (each at half dose) for pre-emptive therapy of cytomegalovirus infection in transplant recipients	133
5.1	Introduction	134
5.2	Material and Methods	135
5.2.1	HCMV surveillance	135
5.2.2	Randomised trial design	135
5.2.3	Statistical analysis	137
5.3	Results	137
5.3.1	Baseline characteristics	137
5.3.2	Assessments of virological responses	138
5.3.3	Assessment of drug toxicity during study period	142
5.3.4	Virologic predictors of the outcome of therapy	144
5.3.5	Quantifying the risk associated with poor antiviral response	150
5.3.6	Subsequent episodes of HCMV viremia	153
5.4	Discussion	155
6	Kinetics of cytomegaloviral load decline in solid organ transplant recipients after pre-emptive valganciclovir therapy	157
6.1	Introduction	158
6.2	Methods	158
6.2.1	Patient selection	158
6.2.2	HCMV monitoring and HCMV quantification	159
6.2.3	Antiviral therapy	159
6.2.4	Calculation of viral kinetics and statistical analysis	159
6.3	Results	159
6.4	Discussion	161
7	Predicting the length of antiviral therapy	167
7.1	Introduction	168
7.2	Methods	169

7.2.1	Patient selection	169
7.2.2	Statistical analysis	169
7.2.3	Definitions	169
7.3	Results	170
7.3.1	Maximum virus load in relation to start of antiviral therapy	170
7.3.2	Baseline and maximum virus load correlates with time to become PCR negative	171
7.3.3	Area under the curve (AUC) before antiviral therapy as a predictor length of viremia	171
7.3.4	Defining diagnostic virus load levels for becoming HCMV PCR neg- ative by 14 days	172
7.4	Discussion	183
8	General Discussion	186
8.1	Diagnosis of HCMV end-organ disease	187
8.2	Treatment with ganciclovir	187
8.3	Treatment with foscarnet/ganciclovir combination	189
8.4	Identifying patients who fail therapy	189
8.5	Markers of cellular immunity	191
	Bibliography	193
	Appendices	232
A	List of manufacturers	233
B	Buffers and cell culture media	234
B.1	R-10 cell culture medium	234
B.2	Media and plates for bacterial cultures	234
B.2.1	Luria-Bertani (LB) Medium	234
B.2.2	Luria-Bertani (LB) agar plates	234
B.3	Buffers for tetrameric complex synthesis	235
B.3.1	Triton wash buffer	235
B.3.2	Urea buffer	235
B.3.3	Refolding buffer	235
B.3.4	BirA buffer	235
B.3.5	FPLC buffer	235
B.3.6	MonoQ anion exchange buffer A and B	236
B.3.7	phosphate buffered saline (PBS, pH 7.4)	236
B.4	Publications	237

List of Figures

1.1	Electromicroscopic picture of HCMV	19
1.2	Genome organization of HCMV	21
1.3	HCMV gene expression during productive infection	28
1.4	Chemical structures of ACV, VACV, GCV and VGCV	43
1.5	Chemical structures of foscarnet and cidofovir	44
1.6	Interaction of HCMV proteins with HLA processing	62
2.1	Synthesis of tetrameric class I complexes	73
2.2	Gating strategy for enumeration tetrameric complex positive cells	76
2.3	Principle Steps of ELISpot assay	79
2.4	Viral load quantification with the TaqMan	85
2.5	Basic model of virus dynamics	90
2.6	Typical virus load profile	91
3.1	Relationship between viral load and owl's eye inclusions	102
3.2	Relationship between owl's eye and virus load	104
3.3	Relationship between owl's eye and virus load	105
3.4	Relationship between owl's eye and virus load	106
4.1	Age distribution of viraemic vs non viraemic patients	117
4.2	Frequency of pp65 specific tetrameric complex positive CD8+ T-cells in non viremic patients according donorrecipient status.	118
4.3	Longitudinal frequency of ppUL83 (pp65) specific tetrameric complex positive CD8+ T-cells in non viremic patients	119
4.4	Longitudinal frequency of pp65 specific tetrameric complex positive CD8+ T-cells in viremic patients	120
4.5	Frequency of pp65 specific tetrameric complex positive CD8+ T-cells	121
4.6	Frequency of pp65 specific PBMCs responding in Interferon- γ secretion	122
4.7	Correlation between the pp65 specific Interferon- γ and the pp65 tetrameric complex frequency	123

4.8	Ratio of the pp65 ELISpot frequency to the pp65 specific tetrameric complex frequency for individual patients	124
4.9	Ratio of the pp65 ELISpot frequency to the pp65 specific tetrameric complex frequency	125
4.10	Probability plot of HCMV viremia	126
5.1	Kaplan–Meier analysis	141
5.2	Relationship between baseline viral load and study outcome	145
5.3	Viral decline rate, after initiating antiviral treatment	146
5.4	Viral replication rate, before initiating antiviral treatment	147
5.5	Correlation between viral decline rate and HCMV baseline viral load	148
5.6	Correlation between viral rate viral decline rate	149
6.1	Decline of HCMV virus load from baseline	165
6.2	166
7.1	Correlation between baseline virus load and maximumvirus load	173
7.2	Scatterdiagram showing the correlation between baseline virus load and time to become HCMV PCR negative	174
7.3	Scatterdiagram showing the correlation between peak virus load and time to become HCMV PCR negative	175
7.4	Relationship between AUC/day before antiviral therapy and time to PCR negative	176
7.5	Scatterplot of AUC before and after initiating antiviral therapy	177
7.6	Relationship between AUC/day before antiviral therapy and baseline virus load	178
7.7	Relationship between AUC/day before antiviral therapy and peak virus load	179
7.8	Dotplot comparing baseline virus load (top panel) and maximum virus load (lower panel). Data are stratified in patients with less and more than 14 days antiviral therapy.	180
7.9	Receiver operating characteristics (ROC) for becoming HCMV PCR negative within 14 days for baseline virus load (top panel) and maximum virus load (lower panel)	181
7.10	AUC/d for primary HCMV infection and reinfection/reactivation	185

List of Tables

1.1	Direct effects of HCMV infection in immunocompromised patients	37
1.2	Indirect effects of HCMV infection in immunocompromised patients	37
1.3	Identified immunogenic pp65 peptides	60
1.4	Identified immunogenic IE1 peptides	60
2.1	HCMV, HHV-6 and HHV-7 specific PCR primers	84
3.1	The number of organs that contained HCMV DNA related to the presence of owl's eye inclusions	101
3.2	The number of organs that contained HHV-6 DNA related to the presence of owl's eye inclusions.	101
3.3	The number of organs that contained HHV-7 DNA related to the presence of owl's eye inclusions.	101
4.1	Demographic data of the study cohort	127
4.2	HLA distribution of the study cohort	127
4.3	Immunosuppressive drugs used in the study cohort	128
4.4	Virological markers of the study cohort	128
4.5	Univariable logistic regression model to identify risk factors associated with HCMV viremia	129
4.6	Frequency of pp65 specific CD8+ T-cells in viraemic and non viraemic patients for different time intervals	130
4.7	Frequency of PBMCs responding after pp65 peptide stimulation with IFN- γ secretion in a ELISpot assay. Frequencies are shown for viraemic and non-viraemic patients	130
5.1	Characteristics of patients according to treatment assignment	139
5.2	Number of patients achieving primary and secondary end-points of the study according to treatment assignment.	140
5.3	Number of patients with reported drug toxicities during the study period, clinical management and study outcome.	143

5.4	Univariable analysis of risk factors associated with primary endpoint becoming HCMV DNA PCR neagtive by 14	151
5.5	Multivariable analysis of risk factors associated with primary endpoint becoming HCMV DNA PCR neagtive by day 14	152
5.6	Frequency of a second episode of HCMV reactivation according treatment allocation.	154
5.7	Frequency of a second episode of HCMV reactivation stratified according HCMV negative at 14 days.	154
6.1	Patients characteristics according to treatment	162
6.2	Virological parameter before starting antiviral therapy	163
6.3	Virological response after initiating antiviral therapy according treatment allocation	164
7.1	Sensitivity and Specificity for becoming HCMV PCR negative within 14 days with a threshold of 10000 genomes copies per mL	182

Abbreviations

ACV	acyclovir
AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphatase
ANOVA	analysis of variance
ATG	anti-thymocyte globulins
AUC	area under the curve
BAL	bronchoalveolar lavage
BMT	bone marrow transplant
BOS	bronchiolitis obliterans
BSA	bovine serum albumin
BSER	brainstem-evoked response
CCMV	chimpanzee cytomegalovirus
cDNA	complementary DNA
CI	confidence interval
CHO	Chinese hamster ovary
CMV	cytomegalovirus
CPE	cytopathic effect
CSF	cerebrospinal fluid
CTL	cytotoxic T cell
CT	computed tomography
DB	dense body
ddI	didanosine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dpi	dots per inch
ds	double stranded
DTT	dithiothreitol
EBV	Epstein Barr Virus

EDTA	ethylenediaminetetraacetate
EIA	enzyme immuno assay
EM	Electron Microscope
ER	endoplasmic reticulum
FAM	6-carboxyfluorescein
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
g	centrifuged force
GCV	Ganciclovir
GI	gastrointestinal
GvHD	graft versus host disease
HAART	highly active antiretroviral therapy
HHV 6	Human Herpes Virus 6
HHV 7	Human Herpes Virus 7
HIV	Human Immunodeficiency virus
HLA	human leucocyte antigen
HCMV	human cytomegalovirus
hpi	hours post infection
HPMPC	Cidofovir
HSV 1	Herpes Simplex Virus 1
HSV 2	Herpes Simplex Virus 2
IFN- γ	Interferon- γ
IL-2	Interleukin 2
IPTG	Isopropyl β -D-thiogalactopyranoside
IU	international units
iv	intravenous
kb	kilo base pairs
kD	kilo dalton
L	liter
Li	lithium
LTX	liver transplant
MMF	Mycophenolate Mofetil
MHC class I	major histocompatibility complex class I
MHC class II	major histocompatibility complex class II
min	minutes
mRNA	messenger ribonucleic acid

nm	nanometer
NS	not significant
ORF	open reading frame
<i>ori</i> Lyt	origin of lytic viral replication
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	streptavidin phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	Foscarnet
pfu	plaque forming units
PM	post mortem
QC-PCR	quantitative competitive polymerase chain reaction
ROC	receiver operator curve
rpm	rounds per minute
s	seconds
SCT	stem cell transplant
SOT	solid organ transplant
TK	thymidine kinase
TMB	Tetramethylbenzidin
VACV	valacyclovir
VGCV	valganciclovir
VZV	Varicella Zoster Virus

Chapter 1

General Introduction

1.1 Human Cytomegalovirus

1.1.1 Introduction

Infection with HCMV is widespread. Most individuals become infected in childhood or early adulthood in less developed countries and late adulthood in developed countries. The virus is transmitted primarily by close contact. Although primary infection, reactivation and reinfection in the immunocompetent is usually asymptomatic, HCMV is a major pathogen in the fetus, transplant patients (solid and bone marrow) and patients infected with HIV. In organ transplant patients, primary infection can occur from the organ graft, or less commonly through blood products. In addition, reactivation of latent HCMV in transplant patients or HIV infected patients can cause clinical symptoms because of the immunocompromised status of the patient. Congenital HCMV infection can cause severe damage to the fetus, with clinical symptoms not necessarily present at birth, but developing in early childhood.

1.1.2 Identification and Classification

At the beginning of the twentieth century, histopathologists frequently found inclusion bodies, later named as owl's eye inclusion bodies, in tissue samples from stillborn fetuses. Later, similar inclusion bodies were found after varicella infection. Until the late 1940s, a unknown protozoan infection was believed to cause HCMV disease and it took further 16 years before three investigators simultaneously discovered the viral origin of HCMV. The name of HCMV was chosen because the virus produces large swollen cell cultures.

HCMV is classified as a member of the *Betaherpesvirinae*, within the virus family of *Herpesviridae* [182]. This classification was initially based on the slow growth in cell culture and the species specificity. The classification was later confirmed by extensive sequence comparison of the HCMV genome with the genomes of other herpesviruses. Sequence analysis, together with restriction enzyme analysis allows the identification of multiple genetic variants (*strains*). Furthermore, with the help of neutralizing antibodies, multiple *serotypes* have been identified. *Serotypes*, are the consequence of the humoral and cellular immune response of an infected patient to a particular HCMV strain, which gives full or partial cross protection to reinfection with a different HCMV strain [294].

1.1.3 Virus structure

1.1.3.1 Virion Morphology

Under the electron microscope, HCMV shows the typical appearance of a herpesvirus, with a capsid diameter of 100 nm (figure 1.1). The virus capsid is surrounded by a lipid envelope. The space between virus envelope and capsid is defined as *tegument*. The capsid consists of 162 *capsomeres*, which enclose the viral deoxyribonucleic acid (DNA). In vitro culture of the virus generates two further particles. The *dense body* (DB), is a large amorphous structure without capsid and DNA, which contains several tegument proteins and envelope glycoproteins. The non-infectious enveloped particle, is an empty capsid with a lipid envelope [234]. Non-infectious particles contain the viral scaffold/assembly protein, which is not present in mature infectious particles. The relative amount of these three forms of HCMV in cell culture depends on the HCMV strain as well as the number of cell passages [182].

1.1.3.2 Structure of the genome

As a member of the herpesvirus family, HCMV has a linear double stranded (ds) DNA genome. With 229354 basepairs (bp) it is the largest genome of the *Herpesviridae*. The genome is divided in two unique regions, designated as unique short (U_s) and unique long (U_l). Each long and short region can be orientated independently from the the other in both directions so four genome isomers can be produced during viral replication. The presence of isomeric forms has no pathologic

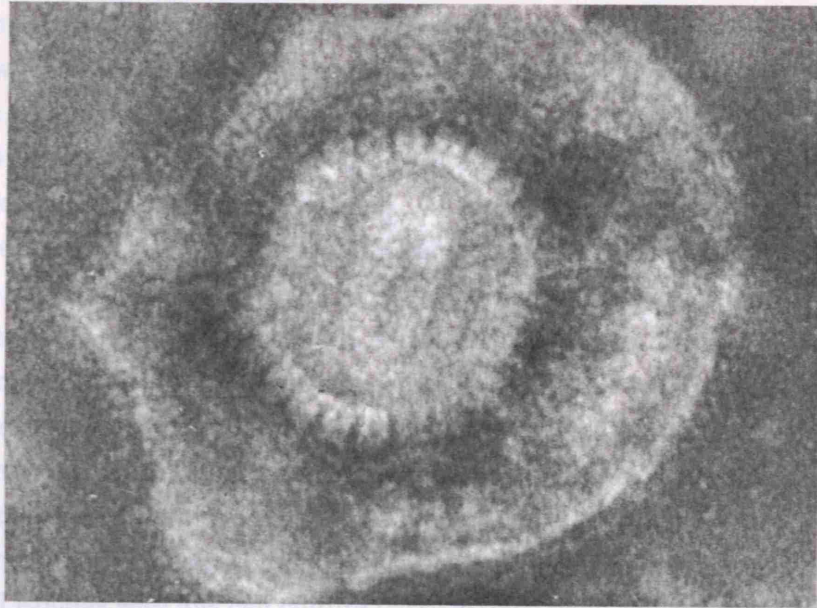


Figure 1.1: Typical electron microscope appearance of cytomegalovirus (Photograph kindly prepared by Mr JA Bishop)

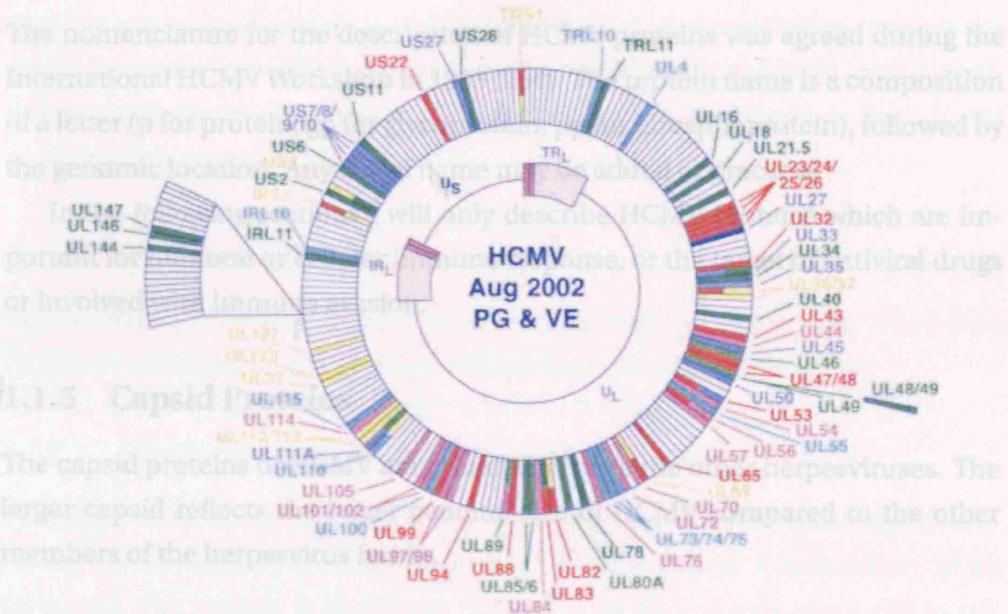
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As a member of the herpesvirus family, HCMV has a linear double stranded (ds) DNA genome. With 229354 basepairs (bp) it is the largest genome of the *Herpesviridae*. The genome is divided in two unique regions, designated as unique short (U_S) and unique long (U_L). Each long and short region can be orientated independently from the the other in both directions so four genome isomers can be produced during viral replication. The presence of isomeric forms has no pathologic or clinical significance. The laboratory strain AD169 was the first HCMV virus whose genome was entirely sequenced [32] and is the prototype for all genomic analysis.

AD169 is a well adapted cell culture virus, which was propagated over multiple cell passages before sequencing. As a consequence of this, AD169 lacks 15 kb of the U_S region [152]. Low passage clinical isolates named *Towne* and *Toledo* have additional ORFs [31]. AD169 lacks in total 22 ORFs which are present in Toledo. Three ORFs missing in AD169 are present in Towne. The missing ORFs might encode important viral proteins which are involved in the pathogenesis of HCMV. For example one of the ORFs missing from AD169 encodes an α -chemokine (U_L146) [204], and another has homology to the herpesvirus entry mediator *Hve A* [168].

Large scale automatic sequencing is widely available and recently several other clinical isolates with low cell culture passage have been sequenced [184]. Computer assisted sequence analysis of AD169 predicted 208 open reading frames (ORF) representing 202 predicted proteins. Some of these proteins map into the terminal repeat regions and are therefore duplicated, which consequently reduces the number of unique proteins to 178. Comparison with the genome of chimpanzee CMV (CCMV) suggests that some of the small ORFs may not encode protein, so the number of ORFs is 145 in AD169 and between 164 and 167 in the wild type HCMV [46].

1.1.4 Important proteins of HCMV and location within the HCMV genome



1.1.5.1 *pUL85* (major capsid protein)

The major capsid protein is a large 1370 amino acid protein, which is highly conserved within the herpesvirus family. It forms major parts of capsid pentamers and hexamers.

The two *pUL85* proteins assemble to form a capsid shell. The capsid proteins are highly conserved within the herpesvirus family. The larger capsid reflects the structure of the other members of the herpesvirus family.

1.1.5.2 *pUL80a* (assembly)

The 765 amino acid protein encoded by the *UL80* ORF is cleaved to produce a serine protease (amino terminal end) and an assembly protein (*pUL80a* (assembly)), by autocatalytic cleavage mediated by a serine-protease.

Figure 1.2: HCMV genome organization. ORFs with identified gene function are filled and the color is assigned according to the gene function. (Figure kindly provided by Prof PD Griffiths and Prof VC Emery)

1.1.4 Important proteins of HCMV and location within the HCMV genome

The nomenclature for the description of HCMV proteins was agreed during the International HCMV Workshop in 1993 [264]. The protein name is a composition of a letter (p for protein; gp for glycoprotein; pp for phosphoprotein), followed by the genomic location. Any trivial name may be added in brackets.

In the following sections I will only describe HCMV proteins which are important for humoral or cellular immune response, or the target of antiviral drugs or involved with immune evasion.

1.1.5 Capsid Proteins

The capsid proteins of HCMV are overall similar to the other herpesviruses. The larger capsid reflects the larger genome size of HCMV compared to the other members of the herpesvirus family.

1.1.5.1 *pU_L86* (major capsid protein)

The major capsid protein is a large 1370 amino acid protein, which is highly conserved within the herpesvirus family. It forms major parts of capsid pentamers and hexamers.

1.1.5.2 *pU_L85* and *pU_L46* (minor capsid protein)

The two less abundant capsid proteins associate in a 2:1 ratio (*pU_L85* to *pU_L46*) to form triplexes, which interdigitate hexons and pentons.

1.1.5.3 *pU_L80a* (assemblin)

The 708 amino acid protein encoded by the *U_L80* ORF, is cleaved to produce a serine protease (amino terminal end) and an assembly protein (*pU_L80a* (assemblin)), by autocatalytic cleavage mediated by a serine-protease.

1.1.6 Tegument Proteins

At least 25 different proteins form the tegument, which fills the space between capsid and envelope [83].

1.1.6.1 *ppU_L83* (lower matrix protein; pp65)

The lower matrix protein pp65 together with the pp150 (*ppU_L32*; basic phosphoprotein) are most abundant proteins made during viral replication. pp65 is the major target for the CD8 T-cell mediated immune response [300], and a component of dense bodies. The protein is also used as the target antigen in the antigenaemia assay.

1.1.7 Envelope Proteins

Studies have shown that HCMV has at least six major glycoproteins. They were originally classified as glycoprotein complexes I, II and III [94].

1.1.7.1 *gU_L55* (glycoprotein B; glycoprotein complex 1)

The major envelope protein in HCMV is gB (*gU_L55*) and is the most highly conserved glycoprotein in mammalian and avian herpesviruses. A homodimer of gB forms gC1. The protein is important in binding to the cell, cell entry, cell to cell transmission and targeting of progeny virus to apical membranes. gB is a major target of the neutralizing antibodies which can be detected in human sera [24]. Two neutralizing epitopes have been identified, termed antigenic determinant 1 and 2 (AD1 and AD2). In humans, the anti gB response is mainly directed against AD1 and to a lesser extent to AD2. Studies have shown that neutralizing antibodies against gB can block virus penetration into cells, but not attachment [185]. The envelope protein gB a candidate as a component of a HCMV vaccine.

1.1.7.2 *gpU_L75* (glycoprotein H; glycoprotein complex III)

Glycoprotein H, is the second abundant envelope protein to which neutralizing antibodies are directed. Neutralizing determinants on gH are less well identified than for gB [33, 223]

1.1.7.3 *gpU_L115* (glycoprotein L, gCIII)

In vitro expression experiments have identified glycoprotein L (*gpU_L115*) as a chaperone protein for the transport of gH to the cell surface [140]. Without gL, gH remains within the Golgi apparatus.

1.1.7.4 *gpUL74* (glycoprotein O, gO)

A candidate gene for the 125-kDa glycoprotein purified from the the gCIII complex. After microsequence analysis, *UL74* were identified as the candidate gene [123].

1.1.7.5 Glycoprotein complex II (gM + gN), gCII

A 18 kilo dalton (kD) protein, derived from the *UL73* gene has been identified to form a disulphide bond complex with the glycoprotein M (*gpUL100* (glycoprotein M)). The complex between the two proteins is named glycoprotein complex II with a molecular weight of 50 to 60 kD. The *UL73* gene product represents the homolog of gN found in other herpesviruses. Neutralizing antibodies against the glycoprotein complex II were found in human sera, which did not react with gM or gN alone [171].

1.1.8 Transactivators

Several ORFs within the HCMV genome can transactivate homologous and heterologous promoters.

1.1.8.1 *pUL122/123*, *pUL82* and *pUL69*

These open reading frame forms the major immediate-early region, the transcribed protein are called IE1 and IE2. The two proteins with a molecular weight of 72 kDa (IE1) and 86 kDa (IE2) act together and release multiple transcription factors that can bind and activate the promoters of β and γ genes. The tegument proteins *pUL82* and *pUL69* activate transcription factors ATF and AP-1.

1.1.9 Genes important for antiviral therapy/resistance

1.1.9.1 *gpUL54*

The *gpUL54* gene encodes for the HCMV DNA polymerase and shows homology to other members of the Herpesviridae polymerases. Several important conserved regions within the protein has been identified which are important for substrate and nucleoside triphosphate binding [59, 34]

1.1.9.2 *gpUL97*

Computer homology searches have identified the *gpUL97* gene as a protein kinase, which was subsequently confirmed by expression of the ORF in E.coli. Extracts of the recombinant protein phosphorylated ganciclovir [162]. The *gpUL97* is the homolog of the thymidine kinase found in HSV or VZV (*pUL97*), however the role of this protein kinase in the HCMV replication cycle is unclear. As with HSV and VZV, HCMV strains carrying mutations in the *gpUL97* gene are less fit compared to wild type [62].

1.1.10 Gene homologs of chemokine receptors or chemokines

Two genes within the U_L region (U_{L33} and U_{L78}) and two genes in the U_S region (U_{S27} and U_{S28}) have been identified as G-protein coupled receptor genes. The protein encoded by U_{S28} can bind the chemokines monocyte chemoattractant 1 (MCP1) and the chemokine RANTES. U_{S28} can act as a chemokine sink, by removing chemokines from the environment of infected cells [286]. The genes U_{L146} and U_{L147} have sequence homology to two α -chemokines. U_{L146} is able to bind to the chemokine receptor CCR2. Both genes might recruit neutrophils during active infection, which become infected and help to disseminate the virus in the body [244, 114].

1.1.11 HCMV replication

The DNA replication of HCMV is very similar to other members of the herpesvirus family. The role of some gene products can be understood by analogy to other herpesviruses, and has been mainly investigated by transient DNA replication assays. In contrast to many other herpesviruses, HCMV does not encode for deoxyribonucleotide biosynthetic enzymes, and hence does not shut off host macromolecular synthesis. The host cells are stimulated to progress into the S phase of the cell cycle, but further progression into the cell cycle is blocked [129], [183], [72], [136]. HCMV has six replication–fork proteins which are well conserved within the herpesvirus family. *pUL54*, a DNA polymerase and its associated processivity factor *ppUL44*, a single strand DNA (ssDNA) binding protein *ppUL57* and a three subunit replicase–primase complex (*pUL70*, *pUL102*, *pUL105*). Viral origin (*oriLyt*) dependent DNA synthesis is initiated by the interaction of U_{L84} with IE2-86. In addition, IE proteins acting as transactivators, are also re-

quired. The linear HCMV genome undergoes recircularisation in the nucleus of an infected cell 4 hours post infection (hpi). DNA synthesis starts by bidirectional ϕ mechanism from a single *oriLyt* [182]. In the late phase, replication switches to a rolling circle mechanism, which is responsible for most of the viral DNA produced during the late stage of infection. This process produces large concatemeric replication units, which are inverted, cleaved to a unit length which can be encapsidated and packed [179].

1.1.12 Herpesvirus cascade

During productive virus replication, the viral genes are expressed in a coordinated cascade (figure 1.3, page 28). The viral genes can be categorized in immediate-early (IE or α), early (E or β) and late (L or γ) according to the time they are transcribed during the viral replication cycle. The HCMV genes are transcribed in the cell nucleus of infected genes by the host RNA polymerase II. Viral gene products may interact with host transcriptional factors, which can stimulate translation of viral genes.

1.1.12.1 Functions of immediate early proteins

The first genes transcribed one hour post infection are the IE genes. the major IE genes are U_L122 and U_L123 (IE1 and IE2). The major IE proteins are required for subsequent viral gene expression and act as transactivators. Cycloheximide, which prevents translation of IE mRNA can prevent viral replication by blocking transcription of the HCMV early genes (see figure 1.3, page 28). In addition the IE genes products interact with several host genes.

1.1.12.2 Function of the early proteins

The E or β genes are only expressed in the presence of functional IE genes but not affected by inhibition of viral replication. The E genes can be further divided in β_1 (E) and β_2 (E-L) genes according the time of expression post infection. The β_1 genes are transcribed 4-8 hours after infection, whereas the transcription of β_2 genes starts at 8 to 24 hours post infection. The E genes encode non-structural proteins such as viral DNA replication factors, repair enzymes and immune evasion proteins [182].

1.1.12.3 Functions of the late proteins

Late HCMV gene products (γ -genes) do not appear before 24 hpi, and require prior viral DNA replication [73]. Late HCMV gene expression can be further divided in γ_1 genes (expressed 24–36 hpi) and γ_2 genes (expressed 24–48 hours). The gene products are mainly structural proteins required for assembly and morphogenesis [182]. Most of the HCMV genes belong to the late class. The transcriptional regulation and the interaction with other viral genes is still poorly understood.

1.1.13 Virion assembly, maturation and egress

Nucleocapsid particles accumulate in the cell nucleus forming 'owl's eye inclusion bodies' in histological sections. Interaction of pU₁₈₆ with the precursor of the scaffolding pU₁₈₀ are formed first in the cytoplasm and later translocated into the cell nucleus. Oligomerization of these complexes leads to hexons and pentons, which interact with pU₁₈₅ and pU₁₄₆ complexes to form a β capsid shell. The viral genome is inserted into this capsid shell and cut by terminase (17). The capsid receives its initial envelope by budding through the nuclear membrane (63). The capsid fuses with the endoplasmic reticulum (ER) and the Golgi apparatus attaches the tegument to the capsid. The mature particles are transported through the Golgi network to the cell surface, where the nucleocapsids and dense body (DB) are released. After 72 hpi, however a substantial amount of virus remains associated with the infected cell.

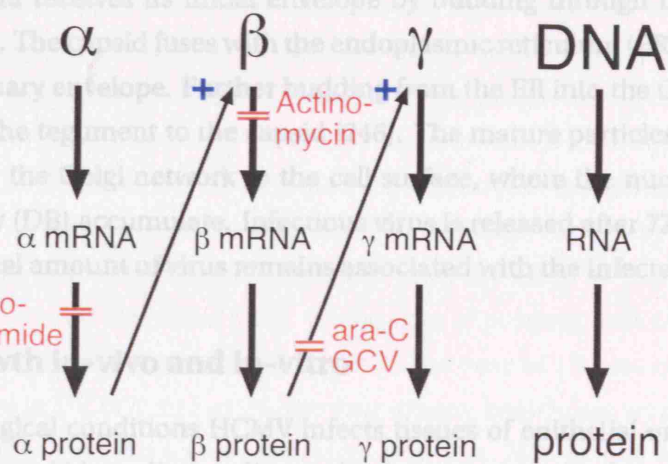
1.1.14 Growth cycle and cell tropism

Under physiological conditions, HCMV infects a variety of cell types of epithelial origin in organs such as lung, kidney, liver, salivary glands, gastrointestinal tract, gut and cells of endothelial origin. In addition, HCMV replicates in nucleated blood cells (leucocytes, macrophages). Initially primary fibroblasts (human primary lung cells) or immortalized fibroblast cell lines were used to propagate HCMV. In recent years commercial life extended cell lines became available which are replacing primary cells.

Figure 1.3: HCMV gene expression during productive infection. Inhibitors of protein synthesis (cycloheximide), transcription (actinomycin D) or DNA replication (cytosine arabinoside (ara-C) or ganciclovir (GCV) can be used to study gene expression and study the viral replication process

complementary DNA (cDNA) into these cells. Further analysis showed that gB interacts with EGFR. This finding however do not exclude the possibility of other cellular receptors or surface structures, like heparin sulphate being involved in viral entry (278). Approximately 5000 binding sites for HCMV are found on the cell surface (278).

Figure 1.3: HCMV gene expression during productive infection. Inhibitors of protein synthesis (cycloheximide), transcription (actinomycin D) or DNA replication (cytosine arabinoside (ara-C) or ganciclovir (GCV) can be used to study gene expression and study the viral replication process



1.1.13 Virion assembly, maturation and egress

Nucleocapsid particles accumulate in the cell nucleus forming 'owl's eye inclusion bodies' in histological sections. Interaction of *pUL86* with the precursor of the scaffolding *pUL80* are formed first in the cytoplasm and later translocated into the cell nucleus. Oligomerization of these complexes leads to hexons and pentons, which interact with *pUL85* and *pUL46* complexes to form a B capsid shell. The viral genome is inserted into this capsid shell and cut by terminase [17]. The capsid receives its initial envelope by budding through the nuclear membrane [83]. The capsid fuses with the endoplasmic reticulum (ER), by which it loses his primary envelope. Further budding from the ER into the Golgi apparatus attaches the tegument to the capsid [246]. The mature particles are transported through the Golgi network to the cell surface, where the nucleocapsids and dense body (DB) accumulate. Infectious virus is released after 72 hpi, however a substantial amount of virus remains associated with the infected cell.

1.1.14 Growth in-vivo and in-vitro

Under physiological conditions HCMV infects tissues of epithelial origin in organs such as lung, kidney, liver, salivary glands, gastrointestinal tract, gut and cells of endothelial origin. In addition, HCMV replicates in nucleated blood cells (leucocytes, macrophages). Initially primary fibroblasts (human primary lung cells) or immortalized fibroblast cell lines were used to propagate HCMV. In recent years commercial life extended cell lines became available which are replacing primary cells.

Epidermal growth factor receptor (EGFR) has recently been identified as a receptor which mediated HCMV virus entry into cells [291]. It was shown that non-susceptible cells became susceptible to HCMV after transfecting the EGFR complementary DNA (cDNA) into these cells. Further analysis showed that gB interacts with EGFR. This finding however do not exclude the possibility of other cellular receptors or surface structures, like heparin sulphate being involved in viral entry [278]. Approximately 5000 binding sites for HCMV are found on the cell surface [278].

1.2 Epidemiology

Infection with HCMV is common in all demographic groups. The relative proportion of seropositive patients depends mainly on age and socioeconomic conditions which also explains geographic differences in seroprevalence. HCMV can be transmitted vertically (mother to fetus) or horizontally (between individuals). In addition, transmission can occur following primary infection, reactivation or reinfection. Prevention of transmission is difficult, because primary infection, reinfection or reactivation are usually clinically asymptomatic and the virus can be shed (saliva, urine, semen) at different times. In the USA, 0.5% to 2% of fetuses become infected with HCMV in utero. A large proportion of children become infected during the neonatal period, by contact with breast milk or saliva. [54, 231, 232, 273]. By the age of 12 months 2% to 10% of the infants are infected. In later childhood, infection occurs commonly in childcare centers by close contact. In addition, in this setting, transmission from the child to the parents or childcare staff has been described [200]. By the time of puberty, 40% to 80% have antibodies against HCMV. A seroconversion rate per year of 1% was observed in pregnant women in UK [97] and of 2% per year among healthcare workers in the USA [55]. Depending on the socio-economic conditions, 70% to 90% of the adult population ultimately become infected with HCMV.

1.3 Transmission of HCMV

1.3.1 Intrauterine infection

Primary HCMV infection during pregnancy is transmitted in utero to the fetus in 37% of cases. Some evidence exists that non transmitting women with primary HCMV infection during pregnancy might have better cell mediated immunity [28]. In only 1% of HCMV seropositive pregnant women transmission to the fetus occurs, presumably by viremia as part of reactivation or reinfection with a different HCMV strain. Only 7% of the newborns, born to a mother with primary infection are symptomatic at birth, which makes it difficult to establish the diagnosis of a congenital HCMV infection in the neonate [271]. Unfortunately, despite being asymptomatic at birth, 90% of these children will develop clinical symptoms within the first two years of life.

1.3.2 Perinatal infection

High viral titers have been measured in maternal genital secretions and breast milk. Ingestion of such infected maternal secretions during labor or from breast milk can cause a primary HCMV infection during the neonatal period [271]. In contrast to intrauterine infection, perinatal infection rarely causes clinical symptoms.

1.3.3 Postnatal infection

The majority of postnatal HCMV infection is asymptomatic. Day care centers in particular have been identified as a place where HCMV is transmitted between children or children to staff members or from children to seronegative parents, with great impact if the mother is pregnant [200], [269]. Primary HCMV infection in young adults might present clinically with a mononucleosis like picture, indistinguishable from Epstein Barr virus (EBV) infection. Studies from organ donors showed that individuals can become infected with multiple HCMV strains [36]. The route of transmission is by sexual contact via saliva or genital secretions.

1.3.4 Blood transfusion

By the mid 1960s, a post blood transfusion syndrome was recognized, consisting of leukopenia, pyrexia and atypical leukocytosis. The syndrome was caused by infection of the transfused patient with primary HCMV [1]. Because attempts to culture the blood borne virus direct from donated blood was not successful, it is believed that latent virus was transmitted by transfused mononuclear cells and reactivated later from these cells after transfusion. With the universal introduction of leukoporesis in blood transfusion medicine, infection of HCMV by blood transfusion has virtually disappeared [157].

1.3.5 Transmission during solid organ transplantation

HCMV infection following a solid organ transplantation is associated with a high mortality if untreated. HCMV seronegative patients (R^-) receiving a solid organ from a seropositive donor (D^+) become infected in 60% to 80% of cases. Natural history studies have shown that these groups of patients experience a more severe form of disease compared to seropositive recipients [216, 207]. Typing of

HCMV strains has proven that seropositive recipients can be infected with a different strain from the donor [105]. Preexisting recipient immunity modulates the disease process but is unable to prevent reinfection (from the graft) or reactivation of latent HCMV. Immunity is modulated by factors such as type of allograft, age, HLA matching, donor recipient serological status and type of immunosuppression [241], [40]. It is still unclear if infiltrating leukocytes or the organ tissue is the source of (re)infection.

1.3.6 After bone marrow transplantation

In allogeneic BMT patients, HCMV infection ranges from 32% to 70%, independent of the serological status of the donor. In contrast to solid organ transplant patients, seropositive bone marrow recipients receiving a bone marrow from a seronegative donor are at highest risk of HCMV infection [238]. Typing of the HCMV in this setting has shown that the virus is derived from the recipient and not from the bone marrow donor. In addition, it has been shown that transfer of cellular immunity from an immune donor can help to control HCMV replication in the recipient [233].

1.4 Pathogenesis and Pathology

The lack of an animal model for HCMV hampered research into how HCMV enters and spreads within the human body. In addition, the absence of clinical symptoms during the early events, when the virus gains access into the body makes it difficult to collect suitable material, which would allow this process to be investigated. Epidemiological data support the view, that HCMV enters the body by mucosal surfaces, either through the genitourinary tract, upper respiratory tract or upper alimentary tract [256]. However, infection of mucosal epithelial cells is not always necessary. It has been shown, that the virus can be transmitted by blood products or by maternal blood from mother to fetus during pregnancy. After initial replication at the site of entry, the virus is disseminated by the haematogenous route. Leukocytes, in particular macrophages, and vascular endothelial cells have been identified as the cells which support virus replication [256]. The haematogenous route then disseminates the virus into different visceral organs. The type of clinical symptoms (if at all) is modulated by the underlying immunological status (see table 1.1). Dissemination of the virus from its

initial replication site is further modulated by viral chemokines, attracting neutrophils and monocytes [204]. Scientific support for a role of viral chemokines in dissemination of the virus comes from mouse models, which showed that mice infected with a virus mutant with a deleted β -chemokine gene have a lower virus load in their organs compared to mice infected with the wild type [243].

1.4.1 Tissue Tropism

HCMV has the ability to infect a wide range of tissues and multiple cell types within an infected organ [209, 256, 178]. The HCMV typical histological changes (eg 'owl's eye' inclusion bodies) have been identified in salivary glands, lung, liver, pancreas, kidney, eye, ear, placenta, alimentary tract, heart, ovaries, pituitary, adrenals, thyroid, brain and skin. Further studies identified salivary glands, bile duct epithelium, bronchial epithelium, islet cells, renal tubular epithelial, astrocytes, neurons and the epithelium cells of the inner ear as the most commonly infected cell types. In addition, more cell types, not showing the typical morphological changes have been identified with more sensitive modern techniques such as immuno histochemistry, *in-situ* hybridization or PCR [281]. Post mortem (PM) studies in non immunocompromised patients have also detected HCMV in apparently normal cells of the kidney, salivary gland, liver spleen and brain [281].

1.4.2 Factors correlating with disease production

1.4.2.1 Type of HCMV infection (primary/reactivation/reinfection)

Primary infection with HCMV has the greatest risk of end organ disease in the immunocompetent and in particular in the immunocompromised host. HCMV infection in the fetus with no developed immune system has a serious effect with uncontrolled virus replication in multiple organs [75]. In SOT patients, reinfection from the transplanted graft represents a greater risk than reactivation of the latent virus, whereas in BMT patients reactivation is associated with a greater risk compared to reinfection from the infused new bone marrow [105, 302].

1.4.2.2 Viremia

Detection of HCMV viremia, either by cell culture, the antigenaemia assay or PCR, has been identified as a risk factor for subsequent HCMV end organ disease.

Several studies have shown a two fold increase in risk, if the virus was detected in saliva or urine, which increased to 5 to 7 fold, if detected in blood. [147, 292]. These important findings also showed that detection of virus in these body fluids precedes HCMV disease by 10 to 14 days and so formed the scientific basis for pre-emptive antiviral therapy [240].

1.4.2.3 HCMV virus load

Virus load was measured for the first time in 1975 by Stagno et al., in urine samples from neonates with proven congenital HCMV infection [272]. Neonates with a more severe form of congenital HCMV infection had a significantly higher virus load than neonates with less severe symptoms. However the difference in virus load was only present within the first 3 months of life [272]. This work showed that virus load correlates with the extent of HCMV disease, and that virus load can be measured at a different site, not necessarily involved in the disease process [98]. Measuring HCMV virus load in saliva, urine or whole blood or serum added another dimension to the qualitative information obtained by conventional PCR or cell culture. It has been shown that increased virus load is an independent risk factor for later HCMV disease [49, 65, 91]. Multivariable logistic regression models in BMT and SOT patients showed that virus load is the most important risk factor for HCMV disease in renal, liver and bone marrow transplant patients. HCMV virus load can now be measured by QC-PCR and more recently by real time PCR using TaqMan or LightCycler based methods.

1.4.2.4 Host factors

The frequency and distribution of HCMV end organ disease varies greatly between the different immunocompromised patient groups (see table 1.1) which suggests an interaction between host factors and HCMV. For example, HCMV can infect bone marrow stromal cells and alter the cytokine secretion profile which in turn, suppresses the proliferation of the bone marrow cells [7, 254]. In addition, HCMV has a direct growth inhibitory effect on early haemopoietic progenitor cells [254]. Both direct and indirect effects of HCMV can explain the myelosuppression after bone marrow transplantation. In contrast to myelosuppression, HCMV pneumonitis, another organ manifestation in BMT patients, may be immune mediated [106]. It rarely occurs in BMT patients before bone marrow engraftment or in HIV infected patients with a very low CD4 count and very

high viral load in the bronchioalveolar lavage (BAL) fluid [268]. In contrast to other immunocompromised patients, HCMV encephalopathy is found more frequently in HIV infected patients. Viremia spreads the virus to the brain where vascular endothelial cells become infected. Astrocytes and neurons become infected later. Histologically the disease presents as microvasculopathy, with or without thrombosis, and as glial nodular encephalitis [287]. Choroid plexus epithelial cells become infected later and as a result HCMV can be detected in the cerebrospinal fluid (CSF). Retinitis, a common manifestation of HCMV in AIDS patients in the pre-highly active antiretroviral treatment (HAART) era has nearly disappeared. The pathological mechanism why retinitis occurred nearly exclusively in HIV patients remains unexplained.

1.4.3 Direct effects of HCMV infection

After blood dissemination HCMV can infect a variety of organs. Direct lysis of the infected cells as a result of HCMV virus replication is responsible for most of the clinical symptoms. The result of intracellular virus replication can be seen histopathological through the formation of owl's eye inclusion bodies. There may be an immunopathological component to some direct effects (eg HCMV pneumonitis and HCMV vitritis following retinitis). In addition, HCMV can be cultured from the infected organs, viral antigen detected by immunohistochemistry or HCMV genome amplified by PCR.

1.4.4 Indirect effects of HCMV infection

The *indirect* effects are the second consequence of infection with HCMV [239]. The indirect effects are distinct from the *direct effects* in two ways: HCMV is not detected in the involved organs using conventional techniques and the evidence for the causal connection with HCMV comes from epidemiological studies or clinical antiviral trials. Indirect effects, associated with HCMV infection are immunosuppression, graft rejection, graft versus host disease (GvHD) and atherosclerosis in solid and bone marrow transplant patients and death in BMT and HIV patients (table 1.2, page 37).

The best evidence for the involvement of HCMV in graft rejection comes from a randomized placebo controlled study in renal transplant patients [167]. The study showed significant increase of graft rejection in patients who received placebo compared to patients who received valaciclovir (VACV). It has been shown that

HCMV can upregulate the expression of human leucocyte antigen (HLA) class I molecules on the cell surface of the renal graft, so potentially making it more likely to be recognized as foreign. A similar process can be seen in liver transplant patients, where graft rejection manifests as a distinct vanishing bile duct syndrome [167, 285]. In heart transplant patients, infection with HCMV is a risk factor for coronary atherosclerosis [166]. This finding was confirmed in a placebo controlled trial, in which a course of 28 days of iv GCV following heart transplantation reduced the risk of accelerated coronary atherosclerosis [284]. Possible mechanisms for HCMV associated coronary graft vasculopathy include increased expression of cell adhesion molecules [155], alteration in the expression of HLA class I or II [121] or dysregulation of wild type p53 [154].

In HIV infection, HCMV may interact with HIV in multiple ways [95]. HCMV is widely disseminated in autopsy tissues and cells or organs are co-infected with both viruses [293, 186]. HIV and HCMV coinfecting patients progress faster to death and HCMV viremia was a stronger adverse prognostic marker than HIV virus load in the pre-HAART era [23, 265].

Table 1.1: Direct effects of HCMV infection in immunocompromised patients

HCMV disease in the immunocompromised (direct)			
Symptoms	Solid Organ	BMT	HIV
Hepatitis	x	x	x
Gastrointestinal	x	x	x
Retinitis	x	x	x x
Pneumonitis	x	x x	
Myelosuppression		x x	
Encephalopathy			x
Polyradiculitis			x
Addisonian			x

Table 1.2: Indirect effects of HCMV infection in immunocompromised patients

HCMV disease in the immunocompromised (indirect) [‡]			
Symptoms	Solid Organ	BMT	HIV
Immunosuppression	x		
Rejection	x	x	
Atherosclerosis	x		
Death		x	x

[‡] Each effect has been significantly associated HCMV in multivariable statistical analysis and/or significantly reduced in placebo controlled trial of anti HCMV therapy

The majority of primary HCMV infections in the immunocompetent host are clinically silent. Occasionally primary infection with HCMV can result in a clinical picture called *mononucleosis like syndrome* which is difficult to discriminate clinically from mononucleosis caused by EBV infection. Clinical symptoms include fever, fatigue, headache, arthralgia/myalgia, lymphadenopathy, splenomegaly and hepatomegaly. The haematologic hallmark is the relative lymphocytosis. The symptoms are more systemic in nature compared to EBV infection. Most frequently, young adults present with this syndrome and it is estimated that 79% of mononucleosis cases are caused by EBV and 21% by HCMV [149]. Low level liver function abnormalities are typical in the HCMV infection, however jaundice or severe hepatitis is rarely seen. The heterophil agglutinin test is negative in contrast to infections with EBV.

1.4.5 Neonates

Only 37% of the primary HCMV infections during pregnancy are transmitted to the fetus and 7% of these have clinically apparent disease at birth [271]. However, of the 93% asymptomatic at birth, 5 to 15% will develop sequelae within the first two years of life. Intrauterine infection of the fetus as part of a reinfection or reactivation of HCMV in the mother is much less likely (0.5% to 1%) and the clinically symptoms at birth are usually milder compared to infants born to a mother with a primary HCMV infection during pregnancy [75]. Primary HCMV infection within the early pregnancy has been associated with a spontaneous abortion rate of 15% [97]. On the other side, naturally acquired immunity (primary HCMV before becoming pregnant) and older age are associated with a reduced risk of congenital HCMV infection [74].

1.4.5.1 Symptomatic at birth

Symptomatic congenital infection is characterized by the involvement of multiple organs. Abnormalities found frequently include hepatomegaly, splenomegaly, microcephaly with or without cerebral calcification, jaundice, petechiae, retinopathy and sensorineural hearing loss [295]. In addition, intrauterine growth retardation and prematurity can be found [21]. Up to 30% of the symptomatic infants die within the first month of life, due to multiorgan disease, hepatic dysfunction, bleeding, disseminated intravascular coagulation or bacterial sepsis [270]. The long term outcome of symptomatic infected infants is poor but highly variable

and usually in favour of children with mild symptoms at birth. Late symptoms include psychomotor retardation, neurologic dysfunction, impaired hearing or deafness [189]. Microcephaly at birth is the most specific predictor of mental retardation and major motor disability, whereas an abnormal CT (computer tomography) at birth is the most sensitive predictor for later disability [189]. Patients with normal hearing both at birth and at follow up are at no greater risk of developing mental impairment subsequently.

1.4.5.2 Asymptomatic at birth

Prospective studies in these patient groups have shown that approximately 15% will develop multiple abnormalities within the first 2 years of life. The most common abnormalities include sensorineural hearing loss, microcephaly, motor defects, mental retardation, chorioretinitis and dental defects [100]. An important late abnormality is the sensorineural hearing loss, which is often bilateral and severe and interferes with communication and learning ability. In more than 80% of the children with this symptom, hearing loss is progressive over the first years of life. Microcephaly develops in 2% to 7% of initially asymptomatic children with the corollary of mental retardation and neuromuscular defects.

1.4.6 The immunocompromised patient

The extent of HCMV disease depends on the underlying disease and the degree of immunosuppression of the patient. The clinical symptoms can range from mild to life-threatening. Fever, up to 40 °C is universally present. It follows a spiking pattern declining below 37 °C. Lethargy, malaise, myalgia or arthralgia are concomitant symptoms.

1.4.6.1 In solid organ recipients

Direct effects

In addition to fever, hepatitis with elevated transaminases and thrombocytopenia can be found. Pneumonitis is an organ manifestation of HCMV especially lung and heart transplantation, which responds poorly to antiviral therapy.

Indirect effects

HCMV infection was a risk factor for atherosclerosis in explanted hearts from patients undergoing heart transplantation in particular in the youngest patient group [53]. HCMV infection post heart transplantation was not only identified as risk factor for graft atherosclerosis, but also identified as a risk factor for more severe graft rejection and graft loss or death [53].

A randomized placebo controlled trial in renal transplant patients, showed graft survival and graft acute rejection was significantly lower in patients receiving high dose of valaciclovir (8g per day for 90 days), compared to placebo. The effect was strongest in the patients with primary HCMV infection (D+R- group) [167], suggesting HCMV is directly involved in the graft rejection process.

In lung transplantation, bronchiolitis obliterans (BOS), an immune mediated process, is a serious cause of graft failure and death. One study identified HCMV infection together with acute rejection or fungal infection as risk factor for developing BOS [84]; another study identified HCMV serostatus together with graft rejection as predictor for BOS [116]. BOS was also reduced with the use of GCV as prophylaxis against HCMV infection in heart and lung transplant patients, supporting the link between HCMV infection and BOS [267, 262].

In liver transplant patients, donor recipient status for HCMV (D+/R-) [82] and HCMV infection were identified as risk factor for invasive fungal infection [39].

1.4.6.2 Bone marrow transplant patients

Direct effects

Pneumonitis as a consequence of HCMV reactivation occurred in 10% to 20% of BMT patients, before introduction of HCMV surveillance and antiviral therapy. The patient presents with fever, hypoxia and the chest X-ray reveals interstitial infiltrates in the lung. HCMV has the ability to infect bone marrow progenitor and stromal cells [7, 89] and so may delay engraftment after bone marrow transplantation.

Indirect effects

Bivariable and multivariable logistic regression analysis in BMT patients have identified HCMV seropositivity as a independent risk factor for invasive fungal infection [280].

1.4.6.3 Patients infected with HIV

Direct effects

Before HAART, 25% of adult AIDS patients developed one or more signs associated with HCMV infection. In contrast to other immunocompromised patients where retinitis is rarely identified, 85% of the AIDS patients with HCMV disease developed retinitis. The patient with retinitis complains about 'floaters' or loss of vision. Ophthalmological examination of the eye shows edema, necrosis haemorrhage and exudate. Later, uveitis, retinal edema and retinal detachment can be seen. Without therapy, patients become blind within a month [22]. Gastrointestinal symptoms, such as odynophagia, esophagitis, abdominal pain and colitis are other end-organ manifestations of HCMV in AIDS patients.

Encephalitis in AIDS patients caused by HCMV can present in two different forms. The first is a disease difficult to differentiate from HIV induced dementia, with symptoms of confusion and disorientation, with the pathological correlate of diffuse damage of the white matter [90]. The second form is due to necrotic ventriculopathy with involvement of cranial nerves. Symptoms include nystagmus and progressive ventriculomegaly, which can be detected by serial CT scanning [135]. HCMV disease has nearly disappeared with the introduction of HAART in 1996.

Indirect effects

HCMV virus load was identified as an disease progression factor independent from HIV load [265]. In addition, HCMV viraemic patients who responded to anti-HCMV therapy had a significant lower risk of developing HCMV disease and a higher rate of survival, independent of HIV virus load [265]. In a multivariable logistic model, detection of HCMV DNA in blood remained a risk factor for death after adjusting for CD4 counts, age and anti-HIV therapy [23].

1.5 Treatment of HCMV infection or disease

1.5.1 Activity of drugs in-vitro

Several antiviral drugs are now licensed in UK and elsewhere for prophylaxis or pre-emptive therapy of HCMV disease. Unfortunately, no single drug has a safety profile that allows it to be used in all different patient groups. In addition,

prolonged prophylaxis may lead to the emergence of antiviral resistance, which makes it necessary to review and change antiviral therapy. In the following sections, I will describe the anti HCMV drugs used currently in clinical practice.

1.5.2 Acyclovir

Acyclovir (9-[2-hydroxyethoxymethyl]guanine) is one of the oldest licensed antiviral compounds (see figure 1.4, page 43).

Spectrum of activity

Acyclovir has the greatest activity against herpes simplex virus (HSV) 1, HSV 2 and varicella zoster virus (VZV). HSV-2 is two fold less susceptible and VZV up to eight fold less susceptible to acyclovir. In addition, acyclovir has activity against HCMV and EBV at higher concentrations.

Mode of action

Acyclovir is selectively phosphorylated (to become acyclovir monophosphate) intracellularly by the HSV or VZV encoded thymidine kinase (TK_v), or the gene product of $UL97$ in CMV. Non infected cells phosphorylate acyclovir 40 to 50 fold less efficiently, which explains the excellent safety profile of this drug [57, 58]. After monophosphorylation, acyclovir is further phosphorylated to acyclovir diphosphate and acyclovir triphosphate by cellular enzymes. Acyclovir triphosphate, which represents the active form of acyclovir, is a competitive inhibitor of the viral DNA polymerase. Acyclovir lacks the 3'-hydroxy group which is essential to form 3'→5' phosphodiester bonds so leads to chain termination after incorporation into the replicating viral DNA chain. In non infected cells, cellular DNA polymerase is 10 to 30 fold less susceptible to acyclovir triphosphate.

Pharmacology

Acyclovir is available as oral preparation, topical cream, ointment and intravenous infusion. After oral administration, only 15% to 30% is bioavailable. A peak plasma level of 10 $\mu\text{g}/\text{mL}$ are achieved after 5 mg/kg iv administration and 0.6/ mL μg after 200 mg of oral acyclovir. Acyclovir is eliminated by renal filtration, which makes dose adjustment necessary in patients with impaired renal function.

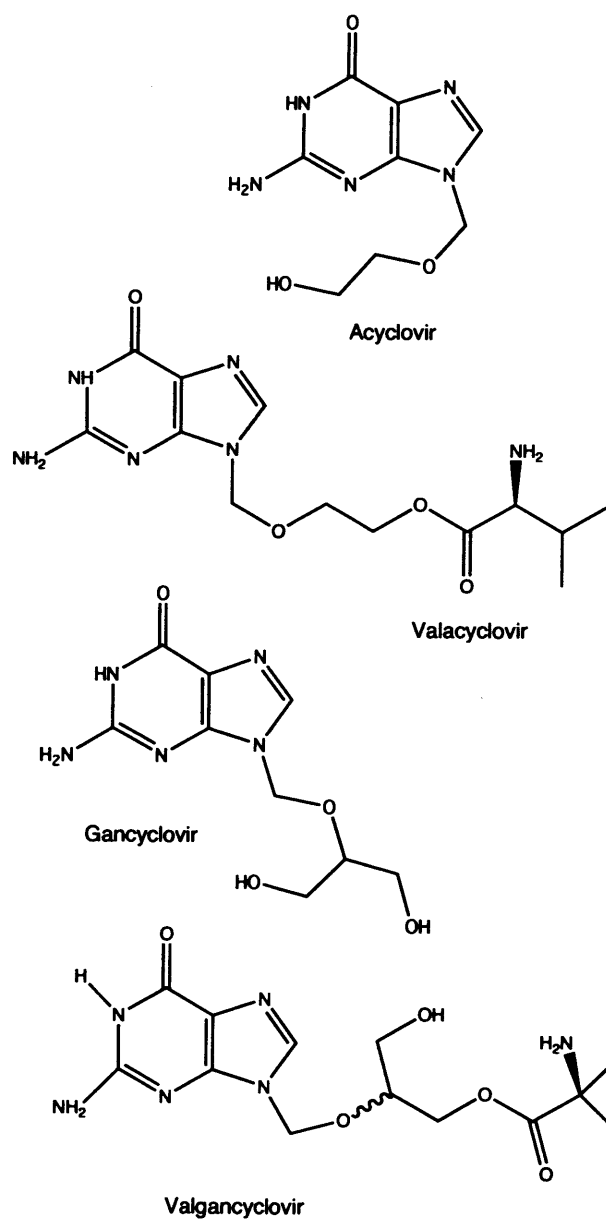
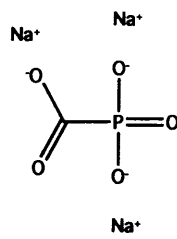
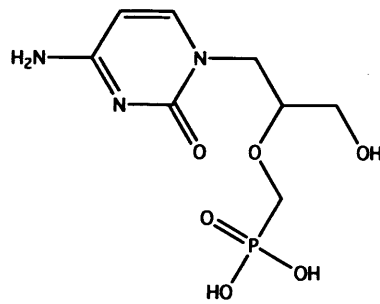


Figure 1.4: Chemical structures of Acyclovir, Valaciclovir, Ganciclovir and Valganciclovir



Foscarnet



Cidofovir

Figure 1.5: Chemical structures of foscarnet and cidofovir

Adverse effects

Acyclovir is one of the best tolerated drugs available although neurotoxicity and nephrotoxicity can occur in patients receiving high doses of acyclovir.

Resistance

Resistance to acyclovir has been reported in patients infected with HSV or VZV, in particular in the HIV infected patient group. Resistance to acyclovir can occur by two different mechanisms, either by mutation within the viral thymidine kinase gene [44], or within the viral DNA polymerase [150]. Mutation of the viral thymidine kinase gene leads to a low affinity for the substrate or to low level enzyme production. In clinical isolates, low level drug resistance exists, even in patients who never received the drug, although with no clinical significance, because these viruses exhibit impaired replication fitness in vivo [203, 242]. Acyclovir TK- resistant isolates are completely cross resistant to valaciclovir, ganciclovir, and famciclovir, but remain susceptible to cidofovir or foscarnet. Virus with DNA polymerase mutations are usually sensitive to foscarnet.

1.5.3 Valaciclovir

Valaciclovir (figure 1.4) is the L-valyl ester of acyclovir [219, 127]. In contrast to acyclovir, which is only 15% to 30% bioavailable, valaciclovir is much better absorbed. Valaciclovir is an inactive prodrug, which is converted in the liver and gastrointestinal tract to acyclovir prior to the intracellular phosphorylation. After oral administration, valaciclovir is rapidly absorbed in the gut and undergoes hepatic hydrolysis, which converts valaciclovir into acyclovir. Valaciclovir has a three to five fold higher bioavailability compared to oral acyclovir. Administration of 2g valaciclovir four times a day (qds) gives similar drug concentrations as 10 mg/kg iv acyclovir three times a day. Once converted to acyclovir, elimination, drug half life, excretion, side effects and drug resistance are as those observed with acyclovir.

1.5.4 Ganciclovir

Ganciclovir (GCV, 9-(1,3-dihydroxy-propoxymethyl)guanine) is another acyclic nucleoside analog of guanosine (figure 1.4). Ganciclovir differs from acyclovir only by the presence of a hydroxymethyl group.

Spectrum of activity

The hydroxymethyl group in the GCV molecule gives GCV improved anti HCMV activity. Inhibitory concentrations are 0.2 to 3 $\mu\text{g}/\text{ml}$ for susceptible clinical isolates. In addition to the anti-HCMV activity, GCV is also active against HSV-1, HSV-2 and VZV. However, the lower safety profile of GCV restricts the use of GCV for the treatment of HSV or VZV.

Mode of action

GCV requires phosphorylation to ganciclovir monophosphate by a viral encoded kinase by analogy to monophosphorylation of acyclovir. In HCMV infected cells, the gene product of UL97 encodes a protein kinase of unknown function, which is able to monophosphorylate GCV [276]. After monophosphorylation, GCV is further phosphorylated by cellular kinases to ganciclovir-triphosphate. GCV-triphosphate is a competitive inhibitor of the HCMV DNA polymerase and can be incorporated into the growing viral DNA strand. However, in contrast to ACV, GCV is not an obligate chain terminator but does stop chain elongation ultimately [71].

Pharmacology

GCV is available in intravenous and oral formulation, but is poorly absorbed after oral administration. For the treatment of HCMV retinitis, GCV is also available as an introcular implant. Only 6% to 9% of the drug is available after oral administration, however an inhibitory drug concentration can be achieved with a drug regime of 1g three times a day. A peak level of 8 $\mu\text{g}/\text{ml}$ can be measured after intravenous infusion of 5mg/kg GCV. GCV is excreted exclusively by renal filtration and 91% of GCV appears unchanged in the urine.

Adverse effects

The most important toxicity associated with GCV is the haematological toxicity [263]. Neutropenia occurred in 40% of patients infected with HIV, and between 7% to 40% in transplant patients [236]. At greatest risk of haematological toxicity are BMT patients. In addition to neutropenia, thrombocytopenia have been reported. Haematologic toxicity is lower after oral administration. Other adverse side effects include, headache, neurotoxic reactions, fever, rash and diarrhoea. In

patients receiving GCV for the treatment of HCMV retinitis, retinal detachment has occurred [252, 141].

Resistance

Two different mechanisms can cause resistance to GCV in HCMV [68]. First, mutation in the U_L97 phosphotransferase gene which is responsible for monophosphorylation of GCV. Most frequently, mutations occur at codon 460, 594 and 595. As expected, these HCMV isolates remain susceptible to foscarnet and cidofovir. Less commonly, mutation in the U_L54 DNA polymerase gene may cause resistance to GCV [68]. Frequently drug resistance against GCV has been observed in HIV patients who have received oral GCV for a prolonged time as prophylaxis against HCMV induced retinitis and in transplant patients [161].

1.5.5 Valganciclovir

Valganciclovir, is the L-valyl ester of ganciclovir and was designed for a similar reason valaciclovir was developed. As a prodrug of ganciclovir, it has enhanced bioavailability, with the same antiviral activity as the the parent drug GCV. The absolute bioavailability of valganciclovir was calculated as 60.9%, more than 10 times the bioavailability of oral ganciclovir. A single dose of 900 mg valganciclovir produces the same drug exposure (defined as AUC of the plasma concentration:time plot) as 5 mg/kg iv GCV [206]. Adverse effects of the drug and resistance profiles are similar to the parent drug GCV.

1.5.6 Foscarnet

Foscarnet (trisodium phosphonoformate) differs from the other anti-HCMV drugs, because it is not a nucleoside or nucleotide analog [288, 289] (figure 1.5). It remains the second most important drug for treatment of HCMV disease and GCV resistant HCMV.

Spectrum of activity

In vitro, foscarnet shows activity against HSV-1, HSV-2, VZV, HCMV, EBV, HHV 6, HIV and HBV [118, 173, 195, 251, 13, 274].

Mode of action

Foscarnet does not require an intracellular phosphorylation step to become activated. It is a noncompetitive inhibitor of the viral DNA polymerase and is not incorporated into the growing DNA chain during viral replication. The viral DNA polymerase becomes inactivated by blocking the pyrophosphate binding site and hence interfering with the pyrophosphate exchange during DNA polymerisation.

Pharmacology

Foscarnet has a very poor oral bioavailability and is therefore only available as iv infusion. Peak plasma concentrations range from 509 μM to 766 μM after infusion of 60 to 90 mg/kg [10]. The plasma half life time is 4.5 to 6.2 h. Renal excretion is the main route of drug elimination, which explains the prolonged plasma half life time in patients with impaired renal function[10]).

Adverse effects

The important adverse drug side effect is renal nephrotoxicity, which can be reduced by pre-hydration and concomitant administration of probenecid. Metabolic abnormalities are the second most common complication of foscarnet. Hypo- and hypercalcemia, hypomagnesemia, hypokalemia and hyper- or hypophosphatemia have been observed and require close electrolyte monitoring of the patient. Neurologic toxicity includes altered sensorium and seizures. In BMT patients, neutropenia and anemia have been reported [130] .

Resistance

Resistance to foscarnet occurs exclusively at the level of viral DNA polymerase. Most mutants remain sensitive to GCV and cidofovir, but cross resistance has been described. These virus isolates had mutations only in the DNA polymerase gene [35].

1.5.7 Cidofovir

Cidofovir (HPMPC) is an acyclic phosphonate nucleotide analog which belongs to a family of phophonylmethoxyalkyl derivatives of purines and pyrimidines [156] (figure 1.5).

Spectrum of activity

Cidofovir was developed as anti-HCMV drug, but has a broad antiherpes activity (HSV,VZV). In addition it shows activity against human papillomavirus [101], polyomavirus [6], adenovirus [47], poxviruses [257] and JC virus.

Mode of action

Cidofovir, structurally equivalent to a nucleoside monophosphate, is taken up in infected and in uninfected cells [119]. Cellular enzymes convert cidofovir to its active diphosphate form. Cidofovir-diphosphate is a competitive inhibitor of the viral DNA polymerase with a 25 to 50 fold greater avidity for the viral DNA polymerase compared to the cellular DNA polymerase. The very long intracellular half life (>48 h), allows intermittent dosing regimes.

Pharmacology

Cidofovir is only available in an iv formulation, topical cream and as intraocular injection. 90% of the drug is excreted in the urine, mainly by tubular secretion, which explains the required dose reduction in patients with impaired renal function.

Adverse effects

Nephrotoxicity, resulting in renal tubular damage is the major adverse effect of cidofovir [215]. Renal toxicity appears first as proteinuria and glycosuria. Cardiomyopathy has been reported in HIV patients if treated simultaneously with Stavudine. Intravitreal complications include iritis and ocular hypotony with the risk of retinal detachment.

Resistance

Resistance to cidofovir may occur after prolonged treatment for retinitis. Mutations within the viral DNA polymerase gene are responsible for the resistance against cidofovir and cross resistance to foscarnet and ganciclovir has been reported [158, 108, 220].

1.5.8 Fomivirsen

Fomivirsen is the first antiviral antisense drug approved and is indicated for intravitreal treatment of HCMV retinitis [48, 126, 102, 103, 104]. Fomivirsen is a phosphorothioate 21mer antisense oligonucleotide, which is only active against HCMV. It is more active against HCMV than is GCV. GCV resistant isolates remain susceptible to fomivirsen.

Mode of action

As an antisense drug, fomivirsen binds specifically to the messenger ribonucleic acid (mRNA) of the immediate-early 2 gene of HCMV which blocks the translation of the mRNA into protein.

Pharmacology and adverse effects

Fomivirsen is only available as intravitreal injection. Increased intraocular pressure and intraocular inflammation are the two most frequently observed adverse drug effects.

Resistance

So far, no clinical HCMV isolate has shown resistance against fomivirsen.

1.5.9 Evidence-base for treatment

The antiviral components described above are used to prevent or treat established HCMV disease in immunocompromised patients. In addition, iv GCV has been used to treat congenital HCMV infection in neonates with neurological symptoms [148]. Three different approaches are currently used in clinical practice, prophylaxis, pre-emptive therapy and treatment of established organ disease.

1.5.9.1 Prophylaxis

Antiviral prophylaxis is defined as administration of the drug before HCMV infection or reactivation is detected. In clinical practice this is usually from the first day of transplantation, or in HIV patients with low CD4 count. In bone marrow transplantation, prophylaxis with GCV is only started after the patient has

engrafted. The length of antiviral prophylaxis has varied from 28 to 90 days in clinical trials.

HCMV prophylaxis in BMT and SOT patients

Prophylaxis with Acyclovir

A double blind placebo controlled study conducted by Prentice et al. showed that iv ACV for 1 month followed by high dose oral ACV for the first 6 months after bone marrow transplantation reduced the risk of HCMV infection and improved survival [217]. This study also showed that high dose ACV is effective in vivo, even if ACV is not very active against HCMV in-vitro.

A randomized, placebo-controlled, double-blind trial of ACV for the prevention of HCMV disease in renal transplant patients, showed a reduction of HCMV disease within the first year [11]. The greatest effect was seen in the high risk D+R- group, where only 16% of the patients had HCMV disease compared to 100% in the placebo group [11].

Prophylaxis with Valaciclovir

In renal transplant patients, high dose valaciclovir (2g four times a day) for 90 days, was able to reduce or delay HCMV disease in seropositive and seronegative patients. In addition, the study found a reduced rate of graft rejection in the seronegative patient group receiving valaciclovir, an indication that HCMV is involved in the graft rejection process [167].

Prophylaxis with GCV

Two placebo controlled double blind studies compared iv GCV vs placebo in the BMT setting [87, 301]. Both studies showed a reduced rate of HCMV infection and HCMV disease in the GCV group, but did not detect a benefit in terms of survival. Patients on GCV with neutropenia had an increased risk of bacterial infection [87].

In heart transplant patients, two placebo controlled double blind studies were conducted. Both studies compared iv GCV vs placebo. Merigan et al found a no reduction in HCMV disease in the seronegative recipient group (primary HCMV infection), but a significant reduction in the seropositive group [180]. On the

other hand, a similar study of Macdonald et al. in 1995, showed a lower incidence rate of HCMV disease in the mismatched group, but no difference in the seropositive patient group [170].

One study addressed the benefit of oral GCV vs placebo in the liver transplant setting [80]. The study found a reduction of HCMV disease in the GCV group, which was seen also in the high risk patient group (D^+ , R^-). The second study compared prophylaxis with high dose of ACV to pre-emptive therapy with iv GCV for 7 days [255]. HCMV shedding was similar in both arms before intervention, but HCMV was less frequent in the intervention arm (ivGCV).

Efficacy and safety of Valganciclovir vs oral ganciclovir in the prevention of HCMV disease in the high risk group (D^+ , R^-) of SOT was investigated in a study by Paya et al. [201]. Patients received either 900 mg Valganciclovir or 3g ganciclovir daily for 100 days post transplantation. Prevention of HCMV disease was similar in both groups at 6 and 12 months. Although HCMV viremia and rate of graft rejection was lower, this was not statistically significant different between the two arms.

All these studies suggest that antiviral prophylaxis against HCMV can reduce the incidence of HCMV disease. However, this is at cost and potential exposure to a toxic drug. In addition, none of these studies addressed the optimal length of prophylaxis.

HCMV prophylaxis in HIV patients

The value of anti HCMV prophylaxis in HIV patients is not clear. One trial, comparing oral GCV vs placebo found a reduced frequency of disseminated disease and retinitis in the treatment arm as well as a statistical longer survival [266]. A similar study conducted in 1998 did not find a reduced incidence of HCMV disease, but a trend towards longer survival [26].

1.5.9.2 Pre-emptive therapy

The term pre-emptive antiviral therapy was introduced by Rubin et al. in 1991 [240]. Pre-emptive therapy is defined as administering anti-viral therapy when infection with HCMV can be detected (cell culture, viral antigen assay or now by PCR) at a body site like blood or urine. The advantage of this approach over prophylaxis is to target the antiviral therapy to patients who show signs of infection and hence are at greatest risk of developing HCMV disease. In addition, this ap-

proach lowers the number of patients exposed to an toxic drug and reduces the chances of developing a resistant HCMV virus when on a suboptimal prophylaxis drug regime. The effectiveness of the concept was first proved in BMT patients who who had a routine BAL at day 35 post transplantation. Patients with HCMV detected in BAL received either iv GCV or placebo for 14 days. Patients who received GCV had a significant lower risk of pneumonia compared to the placebo group [250]. In a similar placebo controlled study, BMT patients received iv GCV or placebo after detection of the virus by culture from blood, urine, throat or BAL. The study showed a significantly better survival in the GCV group [88]. Detection of the virus by PCR is a more sensitive and faster method to guide pre-emptive therapy [56]. Several studies addressed the question which anti HCMV drug prevents HCMV disease in the pre-emptive therapy setting.

Pre-emptive therapy with iv GCV was compared against foscarnet in allogeneic blood or marrow stem cell transplantation. Patients were randomized to receive either iv GCV (5mg/kg twice daily) or foscarnet after detection of HCMV in the blood (antigenaemia or by PCR). Event free survival (defined as HCMV disease) was similar in both groups [229]. A placebo controlled trial in liver transplant patient showed that, pre-emptive administration oral GCV for 8 weeks can prevent HCMV disease in HCMV R⁺ and R⁻ patients [202].

1.5.9.3 Treatment of established disease

Immunocompromised patients presenting with laboratory confirmed diagnosis of HCMV disease have a high mortality, and a highly potent antiviral drug is required to control viral replication and possible immune mediated effects of HCMV disease. A randomized control trial comparing iv GCV vs placebo in BMT patients with biopsy proven HCMV gastroenteritis, showed shortening of virus excretion, but no difference in clinical symptoms, supporting the hypothesis that controlling virus replication is not sufficient once organ disease has been established [227]. GCV was licensed following trials comparing immediate versus delayed therapy for HCMV retinitis in the pre-HAART era.

1.5.9.4 Treatment of symptomatic congenital HCMV infection

The lack of a safe, non toxic highly active anti HCMV drug delayed the investigation of antiviral therapy in symptomatic congenital HCMV infection. In a phase II dose ranging clinical study, iv GCV was administrated for 6 weeks to neonates

with proved symptomatic congenital HCMV infection [296]. Urine HCMV virus load decreased during the time of therapy, but viraemia returned to near pretreatment levels after therapy. Hearing improvement or stabilization was observed in a small proportion of these babies (16%). This study led to a placebo controlled clinical trial comparing iv administration of GCV vs placebo in symptomatic congenital HCMV infection with neurological symptoms [148]. The primary endpoint of the study was improved brainstem-evoked response (BSER) between baseline and at 6 months. The study found a borderline statistically significant improvement at 6 months ($p=0.06$) in the GCV group. However, analyzing the data with prevention of worsening BSER as endpoint showed a highly significant effect of iv GCV ($p<0.01$).

1.6 Detection of systemic HCMV infection

Detection of IgG antibodies in the serum by EIA or indirect immunofluorescence technique can be used to establish the diagnosis of past infection. In addition, detection of IgM antibodies without detection of IgG antibodies is suggestive of a primary HCMV infection, in particular if a convalescent serum shows the presence of IgG antibodies. However, serological methods are unreliable in immunocompromised patients and therefore one of the methods described below is used to detect HCMV in body fluids, organs or blood.

1.6.1 Cell culture

HCMV can be cultured from clinical specimen on HCMV susceptible primary cell lines or transformed cell lines. After 10 to 20 days, the cell culture shows a typical HCMV specific cytopathic effect (CPE). The long culture time required to detect the CPE, together with the low sensitivity makes cell culture not practical for the rapid diagnosis of HCMV. HCMV can be detected earlier by staining the cells, inoculated with clinical specimens, with a fluorochrome labeled antibody (direct or immunofluorescence test), against immediate (IE) early or early (E) antigens.

1.6.2 Detection of HCMV in organs or tissue biopsies

HCMV replication can be detected by histological examination of biopsy or post mortem section. HCMV appears as so called 'owl's eye' inclusion bodies [169,

258]. Immunohistochemistry can be used to enhance the sensitivity [187].

1.6.3 Antigenaemia

The antigenaemia assay, is a method for detection and semiquantification of HCMV leukocyte positive nuclei in an immunofluorescence assay, usually with an antibody directed against the lower matrix phosphoprotein pp65 [93]. The detection of pp65 in leukocytes correlates with the risk of HCMV disease. The advantage of the antigenaemia assay over virus culture or PCR is that the test can be performed within hours. Comparison of antigenaemia against PCR showed a good correlation between both tests in predicting HCMV disease, with a lower sensitivity of the antigenaemia assay [208, 137, 197]. The test is unreliable in neutropenic patients and has disadvantages that samples must be processed within 3 hours to avoid cell lysis [16].

1.6.4 Molecular amplification methods

The polymerase chain reaction (PCR) method is the most commonly used amplification technique to detect HCMV genomic material from blood, tissues or body fluids. The technique can be used qualitatively or quantitatively. Small quantities of clinical material (eg blood or urine) are sufficient for amplification [147]. Prospective studies have also shown that detection of HCMV in blood correlates with the risk of HCMV disease [147]. Quantification of positive samples allows further improvement in the prognostic value of a positive PCR result [65].

1.7 Vaccines

Several strategies have been used in the past to prevent HCMV infection, depending on the patient group. In solid organ transplant patients, HCMV infection can be avoided by transplanting only a seronegative organ into a seronegative patient. However, this approach is not practical with the shortage of solid organs. Prophylaxis with antiviral drugs can prevent primary HCMV infection or reactivation in solid organ transplant patients. Avoiding blood transfusion with blood from seropositive patients or filtered blood products (leukocyte deprived) can prevent HCMV infection. Despite all of these efforts, HCMV infection still persists in immunocompromised patients and in the general population. The ul-

timate solution to HCMV infection would be eradication by active vaccination from the general population. Humoral and cellular immune responses may protect against HCMV disease, so any vaccine may have to target both arms of immunity. Studies have shown that natural HCMV infection mounts a strong immune response against gB (neutralizing antibodies) and pp65 (cytotoxic T cells), which indicates that both proteins are good vaccine candidates. However, natural infection will not protect against re-infection. So that vaccine approaches will be necessary that out-perform the immune responses seen during natural infection.

1.7.1 Live attenuated vaccine

The first attempt to develop a HCMV vaccine was undertaken by Plotkin et al. in the 1970's [210, 221]. Healthy male volunteers were vaccinated with the HCMV strain Towne, which was attenuated by passage in human diploid cells. All seronegative volunteers developed antibodies against HCMV, and the antibody response was boosted in the seropositive group after subcutaneous vaccination. Volunteers vaccinated with the Towne strain were resistant to symptomatic HCMV disease, when challenged a year later with a low passage strain Toledo at 10 or 100 plaque forming units (pfu) [213]. A randomized, placebo controlled study in seronegative renal transplant patients, vaccinated before transplantation, did not prevent HCMV infection post transplantation. Symptoms and severity however were more mild in the vaccinated patient group [211]. The study also showed that no reactivation occurred in the vaccine group who received a kidney from a seronegative donor. A larger study in renal transplant patients showed similar results [212]. The attenuated Towne based vaccine strain is able to induce humoral and cellular immunity in healthy volunteers and patients, but cannot prevent infection. It is now known that the Towne strain lacks 19 genes, present in the clinical HCMV Toledo strain, which may explain the failure of the vaccine to prevent primary infection [31]. For this reason, a recombinant between Towne and Toledo has been made bringing back the 19 missing genes into a Towne background [218], however further laboratory testing is required to prove the safety and immunogenicity of the recombinant virus.

1.7.2 Soluble HCMV gB based vaccine

The HCMV glycoprotein B can be expressed in *E. coli* or human fibroblasts, infected with vaccinia or adenovirus, containing the HCMV gB gene [86, 25, 174]. In fibroblasts expressed gB the protein is postrationally processed and appears with the natural occurring molecular weight in the cell culture supernatant. The expressed protein is recognized by monoclonal and polyclonal HCMV antibodies. Intranasal application of the adenovirus derived gB protein induced neutralizing antibodies in hamsters [174]. None of these expression systems were used to produce the vaccine component used in clinical trials.

A modified HCMV gB protein, containing the entire extracellular glycosylated domain and the intracellular domain, but lacking the transmembrane part of gB was expressed in chinese hamster ovary (CHO) cells and used in a placebo controlled double blinded clinical trial. This HCMV gB protein was given together with a novel adjuvant (MF59) to seronegative volunteers [199]. The vaccine was safe and well tolerated. The antibody response was greater with the new adjuvant, compared to conventional alum adjuvant. The study also provided information about the optimal dose and immunization schedule of this vaccine.

Canarypox is an avian virus, which does not replicate in mammalian cells, but inserted foreign DNA is expressed without virus replication, making the canarypox expression system a potential vaccine delivery system. Phase I trials in mice and guinea pigs, vaccinated with canarypox-HCMV-gB induced a cytotoxic T cell (CTL) and antibody response [85].

Canarypox-HCMV-gB given to healthy volunteers prior to the attenuated Towne based HCMV vaccine, was safe and boosted the antibody response against gB [2]. Furthermore, lipid modification of the amino terminus of the peptide was able to simulate without adjuvant.

1.7.3 Canarypox based pp65 vaccine

Cellular immunity is essential to control HCMV replication, and is mainly directed against the lower matrix protein pp56 (*U_L83*). In a randomized placebo controlled clinical trial, a recombinant canarypox-pp65 was used to vaccinate HCMV seronegative healthy volunteers, at 0,1,3, and 6 months. Cytotoxic T cells against pp65 were detected after two months and persisted for at least 26 months. An antibody response was found against pp65. In addition, PBMCs responded to stimulation with the pp65 peptide in vitro [14].

1.7.4 Peptide based HCMV vaccines

Cell mediated immunity against HCMV is mainly directed against the lower matrix protein pp65. HLA A*0201 restricted peptides were identified in vitro and used to stimulate PBMC from seropositive donors in vitro. In vitro stimulated PBMCs were capable to recognize and kill HCMV infected or peptide-sensitized autologous fibroblasts [50]. Further studies are required to prove the concept of a peptide based HCMV CTL vaccine.

1.7.5 HCMV DNA vaccine

DNA vaccination describes the intramuscular or subcutaneous injection of cloned plasmid DNA under the control of a strong promotor. After injection, the cloned DNA is transcribed and translated into protein and so facilitates a humoral and cellular immune responses [122, 52, 163]. Intramuscular injection of the cloned coding sequence of the pp65 gene into mice produced an antibody response in 60% of the animals [196]. DNA vaccination with two different plasmids, coding for gB and pp65 was able to elicit neutralizing antibodies and a CTL response in mice [66]. Humoral responses elicited by the DNA vaccine were further enhanced by a prime boost approach together with co-immunization of CpG oligodeoxynucleotides and the use of aluminum phosphate gel adjuvant [279]. Clinical trials of safety and efficacy in humans are required for HCMV DNA vaccines.

1.8 Immune response against HCMV

After the primary infection, HCMV remains latent at selected sites [226], under the control of the immune system. Productive replication with transient virus shedding occurs. However, severe infection is limited to patients with impaired T-cell immunity, indicating that the T-cell arm of the immune response provides most protection against HCMV disease.

1.8.1 Humoral immunity

Production of immunoglobulins of the class M appear early after primary infection and can persist for 3 to 4 months. Immunoglobulins of the class G follow several weeks later and persist lifelong. The envelope proteins gB and gH are the

most immunogenic proteins and the target of neutralizing antibodies in the human and in the mouse model [198]. The gB protein is one of the most abundant proteins of HCMV, whereas the surface protein gH elicits a complement independent antibody response. The importance of the humoral immune response is demonstrated by several observations. Mice immunized against murine HCMV gB are protected against a lethal challenge and in the guinea pig model gB antibody prevented congenital HCMV infection [222, 109]. In the transplant setting, administration of HCMV immunoglobulins have shown to modulate HCMV infection [253].

1.8.2 Cellular immune response

The murine CMV (MCMV) model has shown the importance of the cellular immune response. MCMV specific cytotoxic T cells (CTL) are required to recover from MCMV infection. Suppression of the CTL response leads to the dissemination of MCMV and CD8+ CTLs together with natural killer cells are important to prevent recurrence [133, 214]. Adoptive transfer of CTLs protect mice from a lethal challenge with MCMV in the absence of CD4+ T-cells [70]. Adoptive transfer of HCMV specific immunity was first shown in BMT patients. Transfer of the cellular immunity was safe, the activity similar to the that measured in the donor and persisted for 12 weeks [290].

In humans, the major target for CD8+ T-cells against HCMV appears to be the ppU_L83 gene (pp65) [300] followed by the IE1 protein. Over the last years, several HCMV derived peptides have been identified which are recognized by HCMV specific CD8+ T-cells (table 1.3 and table 1.8.2, page 60. Initial studies with limiting dilution assays showed a frequency of 1:5000 CD8+ T-cells directed against the pp65 peptides [20]. Newer techniques, such as tetrameric complexes (see 2.3, page 67) and the ELISpot (2.7, page 77) assay show a much higher frequency in immunocompetent and immunocompromised patients [153, 67, 9, 92, 79, 112].

Table 1.3: Identified immunogenic pp65 peptides

CD8+ epitopes			
pp65 peptide	Start	Length	HLA Type
NLVPMVATV	495	9	A2
VLGPISGHV	14	9	A2
MLNIPSINV	120	9	A2
QYDPVAALF	328	9	A24
VYALPLKML	113	9	A24
RIPHERNGFTV	265	10	B7
TPRVTGGGAM	417	10	B7
EFFWDANDIY	511	10	B12
IPSINVHHY	123	9	B35
YYTSAFVFPTKDVAL	181	15	B35
VFPTKDVAL	187	9	B35
DDVWTSGSDSDEELV	397	15	B35
IHASGKQMWQARLTV	148	15	B52
GKQMWQARLTVSGLA	152	15	B52

Table 1.4: Identified immunogenic IE1 peptides

IE1				
IE1	Start	Length	HLA Type	Reference
YILEETSVM	315	9	A2	[228]
ELKRKMIYM	199	9	B18	[228]
CVETMCNEY	279	9	B18	[228]
DEEDAIAAY	379	9	B18	[228]
EEEEGAQEER	354	10	A2	[78]
VLEETSVML	316	9	A2	[143]

1.8.3 Immune evasion

A feature of all members of the herpesvirus family is the ability to undergo latency. The viral genome persists under these conditions lifelong at specific anatomical sites. The escape from elimination through the host immune system is the result of induction of a latent state, persistence in a privileged site and the expression of genes which interfere with the host immune system (figure 1.6, page 62).

Viral peptides are presented in MHC class I molecules and the infected cell is therefore a target for CD8⁺ CTLs. HCMV unique short region (U_S), encodes for four proteins, which can downregulate the MHC class I complex (U_{S2} , U_{S3} , U_{S6} and U_{S11}). U_{S2} and U_{S11} encode for proteins which redirect the MHC I molecules from the ER into the cytosol where they are degraded by the proteasome, which prevents the appearance on the cell surface [299, 298, 12, 69]. A transporter complex (TAP 1/2, transporter associated with antigen processing) is required to transport proteins into the ER. The viral gene U_{S6} is able to inactivate the TAP 1/2 system by interfering with the TAP1/TAP2/MHC class I/ β_2 -microglobulin complex [4, 117, 160]. The U_{S3} protein causes MHC class I molecules to accumulate in the ER by preventing the transport of the assembled class I molecules to the cell surface [132, 3]. The HCMV glycoprotein U_L16 binds some of the ligands recognized by the NK-activating receptor NKG2D. After infection with HCMV, cells are more susceptible to NK cells, however expression of U_L16 eliminates this effect by binding ULBP1 and ULBP2 [235]. Another mechanism to escape recognition of infected cells by NK cells, is achieved by expression of U_L40 , a glycoprotein containing a nine amino acid sequence homolog of the MHC class I leader sequence. Expression of U_L40 conferred resistance to NK cell lysis via the CD94/NKG2A receptor [282]. The U_L18 gene product might be also involved in escape from NK mediated cell lysis [230, 247].

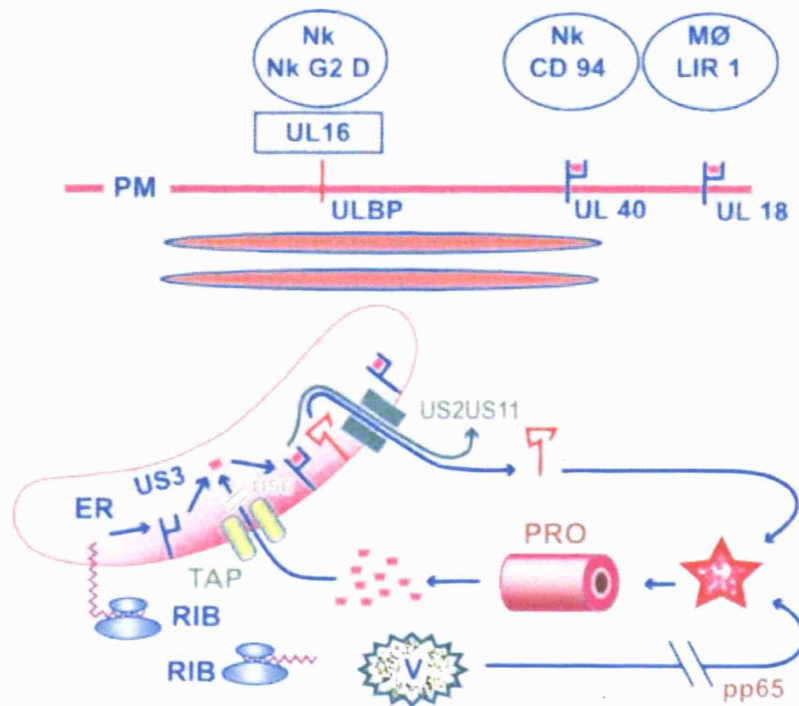


Figure 1.6: Interaction of HCMV proteins with the human HLA processing system
 (Photograph kindly prepared by Prof PD Griffiths and Prof VC Emery)

1.9 Aims of the thesis

The overall aim of the thesis was to investigate the role of HCMV as a pathogen in immunocompromised patients. The first aim was to investigate the role of "Owl's eye inclusions" as a diagnostic marker for HCMV organ disease in HIV infected patients. The second aim was to investigate the T-cell immunity against HCMV post renal transplantation. In particular, if a preexisting immunity against HCMV can prevent HCMV reactivation. Two clinical trials were used to investigate the benefit of adding foscarnet to iv ganciclovir for the pre-emptive treatment of HCMV and to compare iv ganciclovir versus valganciclovir for treatment of HCMV viremia. Finally, the last aim of my thesis was to explore the possibility to predict the length of antiviral therapy which is necessary to become HCMV PCR negative in blood after HCMV reactivation.

Chapter 2

Materials and Methods

All non commercial prepared buffers or media are described in appendix B.

2.1 Isolation of peripheral blood mononuclear cells from whole blood

Whenever possible peripheral blood mononuclear cells (PBMC) were isolated on the same day of blood collection from the patients. If this was not possible, blood samples were stored at room temperature and isolated on the next day. In this case the delay in isolation was recorded. No blood sample older than 48h was processed.

For PBMC isolations, 20 ml of whole blood was collected into blood collection system (Sarstedt), containing Li-heparin (16 IU per 10 ml tube) as anticoagulant. Anticoagulated blood was slowly layered on to Ficoll (Amershan-Pharmacia) using a 1:1 ratio of Ficoll: blood. Overlaying was done by applying the blood sample gently to the side of the sterile 50 ml centrifugation tube (Falcon) already containing 20 ml of Ficoll. The blood/Ficoll was centrifuged at 800 g (2000 rpm, Haereus benchtop centrifuge) for 20 min at room temperature without a brake. Red blood cells and neutrophils are pelleted through the Ficoll to the bottom and PBMCs are concentrated at the Ficoll plasma interface, visible as a milky white band. PBMCs were aspirated from the interface with a 10 ml pipette and transferred into a fresh 50 ml centrifugation tube. PBMCs were washed in pre-warmed RPMI-1640 medium and subsequently pelleted by further centrifugation (600 g; 15 min, room temperature). The supernatant was discarded and the PBMC pellet resuspended in RPMI-medium. PBMCs were washed three times to remove all remaining Ficoll before cryopreservation. Before the last wash, an aliquot of 10 μ L for counting and assessing the cell viability (see section 2.1.1). After the final wash, isolated PBMCs were resuspended in fetal calf serum (FCS) to obtain a cell concentration of 6 to 10×10^6 /ml (twice of the final freezing concentration and kept on ice for 20 min to reduce cell clumping during the freezing process.

2.1.1 Counting cells and assessing cell viability

For cell counting, 10 μ L of cell suspension were mixed with 10 μ L of a 0.2% Trypan blue solution (Sigma) and transferred in a Neubauer chamber (haemocytometer). Live cells (bright) and dead cells (violet) in the outer 16 squares were

counted. The total number of isolated PBMCs were calculated.

2.1.2 Cryopreservation of peripheral blood mononuclear cells

A freezing solution was prepared containing 80% FCS and 20% DMSO (dimethylsulfoxide, Sigma). The freezing solution was kept on ice for 10 min. Isolated PBMCs (see section 2.1) were mixed with the freezing solution in a ratio of 1 : 1 (v/v), achieving a final concentration of 10% DMSO. Cells were transferred in 1 ml cryogenic vials (Alpha Laboratories) and placed in pre-cooled gradual freezing chamber (Nulgene). The freezing chamber allowed a controlled freezing process if placed in a -80 °C freezer for at least 24 h. Frozen PBMCs were transferred into liquid nitrogen container for long term storage.

2.2 Thawing of Lymphocytes

Cells for thawing were transferred from the liquid nitrogen tank to the -80 °C freezer 24 h before thawing. Cryovials containing the PBMC cells were quickly transferred from the freezer into a 37 °C water bath. Homogenous quick defrosting was achieved by gentle shaking in the water bath. After thawing PBMCs were transferred into a sterile universal tube (Sarstedt, Newton, NC, USA) containing 10 ml of R10 medium. Cells were washed twice in 10 ml R10 medium (medium exchanged by pelleting the cells by centrifugation, 10 min 600g). PBMCs were finally resuspended in 5 ml R10 and incubated for 3 to 4 hours at 37 °C at an CO₂ incubator to recover. The thawed PBMCs were counted (see section 2.1.1 and cell concentration adjusted for the different experiments (see section 2.7 and 2.4).

2.3 Construction of HCMV HLA peptide tetrameric complexes

A summary of the steps in construction of HCMV HLA peptide specific tetrameric complexes is shown in figure 2.1.

2.3.1 Peptides

Four different HCMV specific tetrameric complexes were constructed for the HLA types A*002, B*007, B*008 and B*3501 as described previously [5, 190]. The fol-

lowing HCMV HLA restricted pp65 specific peptides were used:

NLVPMVATV	HLA A2 restricted
TPRVTGGGA	HLA B7 restricted
DANDIYRIF	HLA B8 restricted
IPSINVHHY	HLA B35 restricted

Peptides were synthesized by a commercial company (Invitrogen).

2.3.2 Recombinant E.coli

E.coli strains, transformed with an expression vector pET (Novagen), containing the modified genes for the heavy chains of the HLA A*0201, B*0702, B*0801 and B*3501 and for the β_2 -microglobulin under the control of the T7 promoter were kindly provided by F. Lechner, University of Oxford. The HLA heavy genes were modified at the C-terminus, by insertion of a biotinylation site sequence (LGGIFEAMKMEIRD) as described previously [29, 249]. From each recombinant E.coli strain, glycerol stocks were prepared and stored at -80 °C .

2.3.3 Expression of β_2 -microglobulin and HLA heavy chains in E. coli

Transformed E.coli (see section 2.3.2) were streaked onto a LB agar plate (with 100 $\mu\text{g}/\text{ml}$ ampicillin and 34 $\mu\text{g}/\text{ml}$ chloramphenicol). The following morning a single colony was inoculated into 1L LB-medium (with antibiotics ampicillin and chloramphenicol) and incubated at 37 °C with vigorous shaking (240 rpm). Thirty milliliters of this pre-culture was transferred into several 4L flasks (filled with 1L LB-medium). Bacteria cultures were incubated (shaking, 350 rpm) and bacteria growth monitored by optical density method (ΔOD_{600nm}). An aliquot of 1 ml (pre-induction sample) was collected and stored at 4 °C for comparison with post-induction sample. When the bacterial cultures were in the logarithmic growth phase ($\Delta\text{OD}_{600nm}=0.5-0.7$), 0.5 ml of 1M β -D-thiogalactopyranoside (IPTG) was added to each 1L culture. Bacterial cultures were incubated for a further 5 to 6 hours. A post induction sample was taken and examined together with the pre-induction sample in a 10% acrylamide gel electrophoresis, using the mid-range protein molecular weight marker (14.4 kD to 97.4 kD) to identify the appropriate bands.

2.3.4 Inclusion body purification

The bacteria were spun down by centrifugation (4000 rpm, 30 min, 4 °C). The pellet was resuspended in a total of 50-75 ml ice cooled PBS. The resuspended bacteria pellet was sonicated in bursts of 30-60s with cooling on ice between each burst, to avoid degradation associated with heating secondary to sonication. Sonication was continued until the samples poured like water (5 to 10 bursts). Sonicated samples were centrifuged at 4 °C Sorvall SS34 rotor, 15000 rpm, 10 min). After centrifugation, the inclusion bodies (with the expressed protein) is pelleted to the bottom of the centrifugation tube, with impurities and unsonicated bacteria layered above. The supernatant was discarded and the inclusion body pellet thoroughly resuspended in Triton wash buffer with a homogeniser. Washing and homogenizing steps were repeated three times. washed three times with Triton wash buffer (with centrifugation steps between). After the third washing step, the inclusion body pellet was homogenized in 10 to 15ml urea buffer (see B.3.2). To aid the dissolving of the inclusion bodies, the solution was left overnight at 4 °C under gentle agitation. The dissolved protein was collected from the supernatant after centrifugation (15000 rpm, 10 min, 4 °C). A small aliquot was used to measure the protein concentration (BioRad, DC protein assay) and to assess the purity of the protein by polyacrylamide gel electrophoresis. The protein was stored at -80 °C (in 30 mg protein aliquots) until needed.

2.3.5 Refolding of monomeric HLA-peptide synthetic molecules

Individual HLA heavy chains (A2,B7,B8 and B35) were refolded with the purified β 2-microglobulin protein. Thirty milligrams of each protein and 10mg of the relevant HLA restricted peptide (see section 2.3.1 (diluted in 100 μ L DMSO) were added to 500ml of ice cold refolding buffer (appendix) with stirring at 4 °C for 48 h. The refolded products were concentrated to a volume of 5 ml (1:100) by ultrafiltration with an Amicon stir cell concentrator (cut-off pore size 10 kD).

2.3.6 Biotinylation of HLA-peptide monomers

After concentration, the buffer was exchanged, by passing the concentrated and refolded tetramer complex through a PD-10 column (Amershan-Pharmacia). The PD-10 column was first equilibrated with 25 ml BirA buffer (5 fold of the column volume), loaded with 2.5 ml of the tetramer complex and eluted with 3.5 ml BirA

buffer. Protease inhibitors leupeptin and pepstatin (Sigma) were added (final concentrations 1 $\mu\text{g}/\text{ml}$). D-Biotin (100 μL ; 100 mM, Sigma) were added together with ATP (500 μL , 100 mM, Sigma) and BirA (15 μL , 50 μM , Sigma). The solution was incubated at room temperature overnight. Precipitate was removed by centrifugation (13000 rpm, 4 $^{\circ}\text{C}$, 5min) prior to FPLC purification (see section 2.3.7).

2.3.7 FPLC purification of the refolded biotinylated monomers

Biotinylated protein complex was further purified by FPLC gel filtration through a Superdex-75 (Amershan-Pharmacia) column. The column was equilibrated with 500ml of FPLC buffer, sample (7.5 ml) loaded with the help of a 10ml loop, and filtrated through the column with 4 ml/min. The protein concentration was continuously monitored and the elute collected in 5 ml fractions. The 42 kD to 45 kD protein peak was pooled and kept on ice until the next purification step.

2.3.8 Ion exchange purification of the selected FPLC fraction

The pooled protein peak (see 2.3.7) was further purified by ion exchange chromatography on a monoQ anion exchange column (BioCad system, GMI). Anion exchange column was equilibrated with monoQ buffer A (low salt), the sample loaded, unbound material washed off with buffer A (20 ml) and then eluted with an gradient of 0 to 100 % monoQ buffer B. The entire gradient runs over 20 ml. Protein concentration was monitored throughout the elution process and the elute was collected in 1ml fractions which were tested in a EIA (Enzyme immuno assay) for biotinylation (see section 2.3.9).

2.3.9 Protein complex conformation EIA

Simple measure of the protein concentration does not give any information about how much protein has correctly refolded. To assess the refolding and purification process, a conformation specific EIA was performed.

A 96 well microtiter plate was coated with the antibody W6/32 (100 μL /well, 5 μg /ml in PBS, Sigma), which recognizes only correctly folded HLA-I molecules. The coated plate was kept for 3h at 37 $^{\circ}\text{C}$, antibody solution removed and the plate blocked by adding 100 μL of PBS/1 % BSA (v/w). The microtiter plate was kept overnight at 4 $^{\circ}\text{C}$ and washed six times with PBS before use. A 1:10 dilution

of the ion exchange fractions (50 μL) were added and serially diluted 3 fold down each lane. The plate were incubated at room temperature for 1h. Samples were removed, and the microtiter plate washed six times with PBS. Fifty microliters of a 1:10⁴ rabbit anti-human β 2-microglobulin (Dako) antibody were added to each well and incubated for 20min at room temperature, followed by 6 washes with PBS. A 1:10⁴ of AP (alkaline phosphatase) conjugated to anti-rabbit IgG (dilution in PBS) were added, and incubated for 20min at room temperature. The microtiter plate was washed six times with PBS and developed with the colometric assay kit for AP (BioRad) and read in a spectrophotometer at OD_{600nm}.

2.3.10 EIA to test for biotinylation

A 96 microtiter plate was coated with 100 μL of a 1:20 (in PBS) dilution of the collected fractions from the anion exchange chromatography and incubated at 37 °C for 90min or at 4 °C overnight. The plate was washed six times with PBS and 100 μL of a 1:1000 dilution of extravidin-peroxidase conjugate (in PBS/10 % BSA (v/w), Sigma) were added to each well and incubated at room temperature for 20min. The microtiter plate was washed (six times in PBS) and the TMB (Tetramethylbenzidin, Sigma) substrate was added and the reaction left to develop. Positive, biotinylated fractions, turned blue.

2.3.11 Concentration of correctly folded and biotinylated protein fractions

Fractions identified positive in both EIAs were concentrated by spinning through a centrprep-10 (Amicon). The protein concentration was determined in the final concentrated sample which was stored at -80 °C until needed.

2.3.12 Tetramer formation with Streptavidin

Monomeric HLA-I/ β 2-microglobulin complexes were tetramerized by adding phycoerythrin labelled streptavidin. First, the mass of the total streptavidin required to saturate all biotin binding sites at a 1:4 molar ratio (streptavidin:refolded complex) was calculated (streptavidin required = total mass of biotinylated refolded complex by 3.16). Next the volume of streptavidin solution which was necessary was calculated (0.2 mg/ml streptavidin-phycoerythrin (PE), Sigma) and divided by 10. At hourly intervals a 1/10 aliquot of streptavidin was added, and rotated

slowly (< 10 rpm) at 4 °C . The biotinylation EIA (see section 2.3.11) was used to test for tetrameric complex formation. The samples should become gradually negative for biotinylation, as the exposed biotin is occupied by streptavidin. Correctly folded, biotinylated and tetramerized samples were kept in the dark at 4 °C

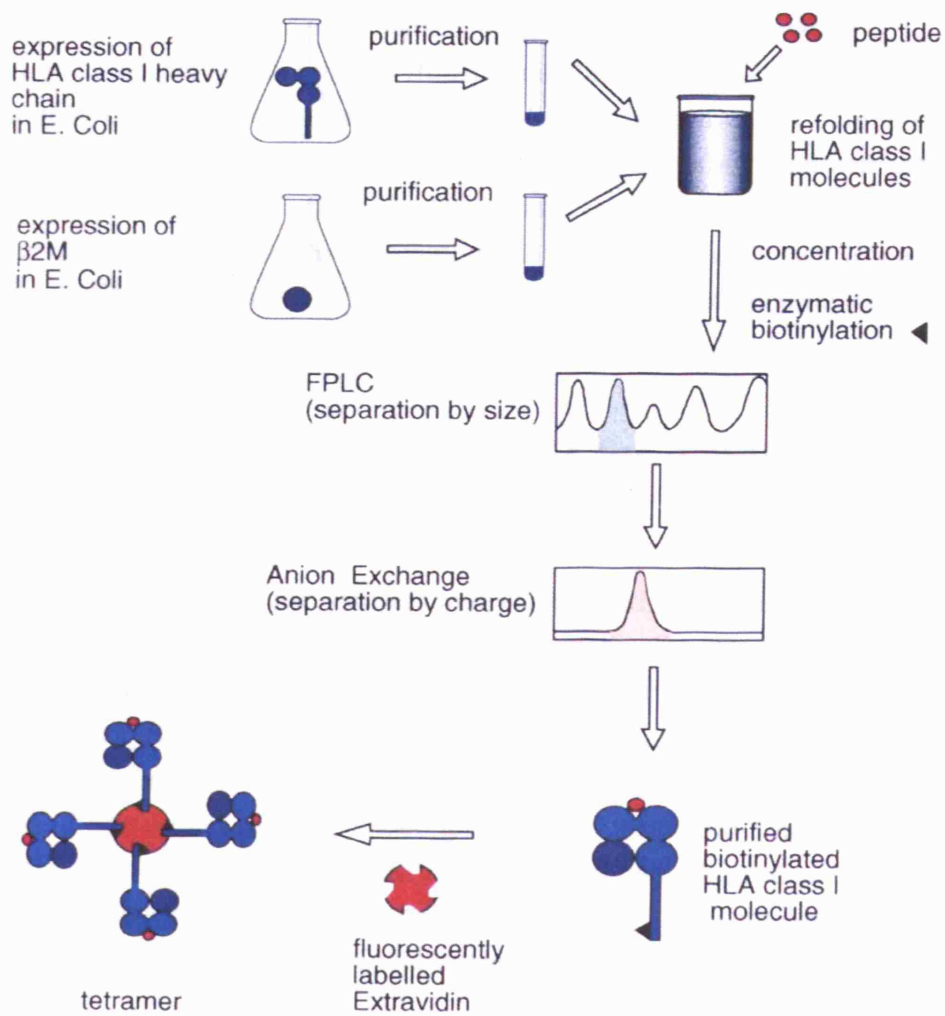


Figure 2.1: Synthesis of tetrameric class I complexes. Figure adapted from Lechner et al.[159]

2.4 Immunostaining with HCMV pp65 tetrameric complexes

For HCMV pp65 tetrameric complex staining, 1×10^5 to 3×10^5 PBMCs were transferred into a FACS tube (Becton Dickinson) and washed once with 4ml RPMI medium. PBMCs were pelleted by centrifugation (600g/5min, room temperature). The supernatant discarded and the cells resuspended in the remaining RPMI medium (usual 50 μ L). One microliter prediluted HLA specific tetrameric complex was added and incubated at 37 °C for 30min. PBMCs were either fixed with 200 μ L 2% PFA or stained with further cell surface markers (CD8, see section 2.5. In the latter case, 4 ml PBS/Az (PBS containing 0.1 % sodium azide (v/w)) was added and samples were divided in four different FACS tubes and staining for several activation markers were performed.

2.5 Immunostaining of PBMCs for lymphocyte surface markers

Tetrameric complex stained samples were stained with CD8 (PerCP labelled, Becton Dickinson) alone or together with one of the following FITC labeled memory or activation surface marker (CD28, CD38, CD45RO or HLA-II DR, all Dako). To the cell suspension (50 μ L) 5 μ L PerCP labeled CD8 antibody were added and if necessary 3 μ L of either CD28, CD38, CD45RO or anti HLA-II DR (all FITC labeled). The samples were incubated for 30min on ice (without light exposure), washed once with PBS/Az and cell pellet resuspended. Stained samples were fixed by adding 200 μ L of 2 % PBS/paraformaldehyde. Fixed samples were stable for several weeks if stored at 4 °C without light exposure.

2.6 Aquisition of data and flow cytometric analysis

Data were acquired on a four channel FACSCalibur System (Becton Dickinson) with CellQuest (Becton Dickinson) acquisition and analyzing software. At least a total of 100000 events were aquired, or until sample was finished. Samples with less than 20000 events in the lymphocyte gate were excluded from the statistical analyzes.

2.6.1 Gating strategy and for enumerating HCMV pp65 specific CD8 T-cells

All samples were analyzed with CellQuest software (Becton Dickinson). An example is shown in figure 2.2 on page 76. A forward and sideways scatter diagram was used to identify the lymphocyte population and a region drawn around this population (R0), (see figure 2.2, upper left panel). CD8+ T-cells (R1) were subsequently identified after gating (G0) on the lymphocyte region (R0) with a forward scatter diagram versus FL3 (FL3=channel for PerCP fluorochrome, 2.2, upper left panel). The tetrameric positive CD8+ T-cells were either identified and enumerated in a FL3 (PerCP) versus FL2 scatterplot (FL2 channel for PE), gated on lymphocytes and CD8+ cells (R0 and R1, figure 2.2 lower right panel) or in a scatter diagram FL3 versus FL2 (gated on R1, figure 2.2 lower left panel). The former were used to calculate the percentage of tetrameric positive cells within the CD8+ cell population, the latter to enumerate the CD8+ tetrameric positive cells within the lymphocyte population.

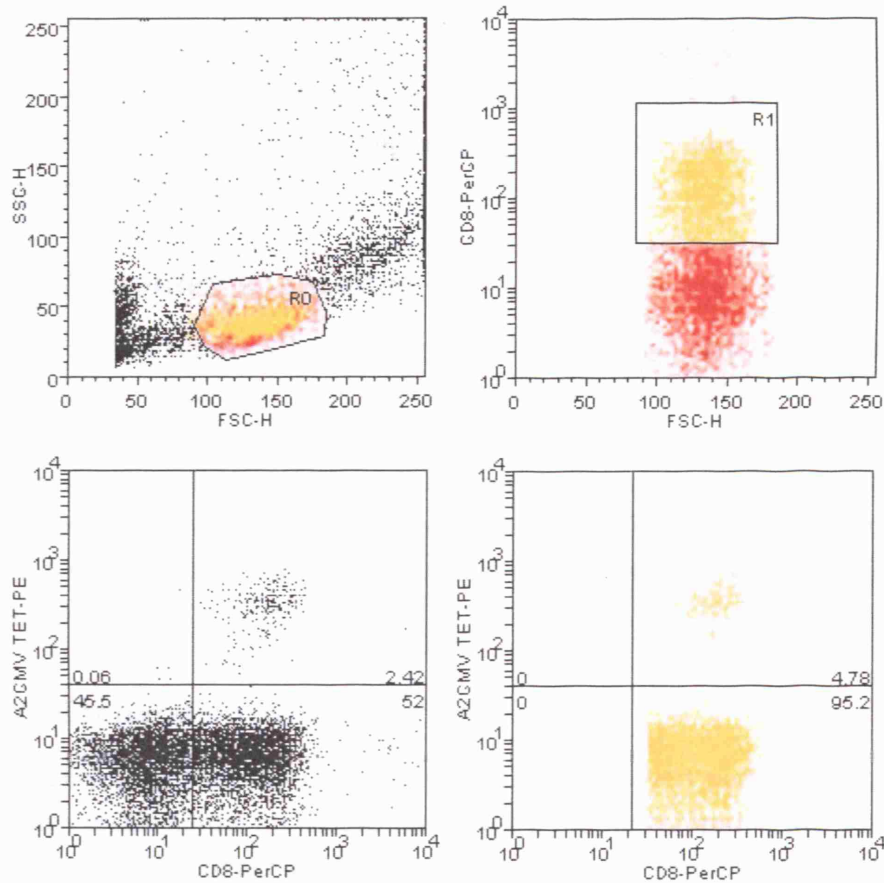


Figure 2.2: Gating strategy used for enumeration of pp65 specific CD⁺ T-cells with tetrameric complexes. Upper left panel: Forward versus Sideward scatter diagram, R0=Lymphocyte gate. Lower left panel: Scatterdiagram FL2 (PE fluorochrome) versus FL3 (PerCP fluorochrome) gated on lymphocytes. 2.47% of lymphocytes are pp65 tetrameric complex positive. Upper right panel: Forward scatter versus FL3, to identify CD8⁺ T-cells (region 1 (R1)). Lower right panel: Scatterdiagram FL3 versus FL2, to identify pp65 tetrameric complex positive T-cells within the CD8⁺ population (R1). In this example, 4.78% of CD8⁺ T-cells are pp65 tetrameric complex positive. See section 2.6.1, page 75 for more explanation

2.7 CD8 ELISpot assay

The ELISpot assay is a simple and sensitive method to identify cells which are functionally capable to secrete interferon- γ (or other markers, like TNF-*alpha* or interleukin 2) after peptide stimulation [43, 42]. In addition, depending on the assay conditions and peptide used, the ELISpot assay allows the enumeration of activated CD4 or CD8 T-cells. In the following section I describe the CD8 specific ELISpot assay (see figure 2.7).

2.7.1 Microtiterplate coating

Twenty four hours before the assay, a 96 well microscreen plate (Millipore) was coated under sterile conditions with 50 μ L (per well) of a 1:66 dilution (in PBS) of anti-human IFN- γ antibody (1-D1K, Mabtech). The microscreen plate was left overnight at 4 °C . The coating antibody was discarded and the microscreen plate was washed six times with filtered PBS (0.2 μ M). To each well, 100 μ L of R10 medium was added to block non-specific binding sites (3-4 hours at room temperature).

2.7.2 Preparation of antigens and cells

Thawed cryopreserved PBMCs were counted after a recovery period of 3 hours at 37 °C in a tissue culture incubator. If necessary, the concentration was adjusted to 5×10^4 or 1×10^5 R10 per 90 μ L . Ninty microliters cells were added to each well together with 10 μ L (200 mM stock in RPMI) relevant peptide (in triplicates) or 10 μ L R10 for the negative controls (in duplicates). In addition, one sample well contained 5 μ L of a 1mg/ml PHA solution as positive control. The ELISpot plate was incubated for 16h at 37 °C /5% CO₂.

2.7.3 Developing ELISpot plate

The cells in the microscreen plate were removed and the plate washed 4 times with PBS containing 0.05% Tween-20 (Sigma), followed by two further washes with filtered PBS. Fifty microliters of a 1:1000 dilution (in PBS/0.5% FCS) of an biotin labeled anti-interferon- γ antibody were added (7-B6-1-biotin, Mabtech) to each well and incubated at room temperature for three hours. ELISpot plate was washed 4 times with PBS/0.05% Tween-20 and twice with filtered PBS. Sub-

sequently, 50 μL of a 1:1000 dilution (in filtered PBS) of streptavidin-AP (alkaline phosphatase, Mabtech) were added and incubated at room temperature for a further two hours, followed by 6 washes (4 times PBS/Tween-20, twice with filtered PBS), to remove all unbound streptavidin. The ELISpot plate was developed with the BioRad AP substrate kit (BioRad). The reaction buffer was diluted 1:25 in water (for one plate 200 μL buffer in 4.8 ml water). Reagents A and B were diluted 1:100 in with the diluted reaction buffer (50 μL A and B in 5ml 1:25 diluted reaction buffer). Fifty microliters were dispensed into each well. Spots developed blue within 20 min at room temperature. The reaction was stopped by washing with tap water. The ELISpot plate was dried overnight and read with a commercial ELISpot reader.

2.7.4 Analysis of ELISpot plate

The ELISpot plate was read with an automatic ELISpot reader. The image of each well was inspected by eye to remove artifacts which were not due to interferon- γ secretion. The automatic reader enumerates the number of spots in each well.

2.7.4.1 Calculating the number of responder cells

The number of PBMC's responding to peptide stimulation was calculated as followed. The mean number of positive cells in the negative wells (without peptide, background) were deducted from mean number of spots in the positive wells (with peptide, triplicates) and expressed as percentage of PBMC's responding to peptide stimulation by interferon- γ secretion.

2.8 Extraction of viral DNA from whole blood or paraffin embedded tissue samples

A commercial DNA extraction kit (QIAamp DNA mini kit, Qiagen) was used to extract DNA from whole blood and paraffin embedded tissue samples. Extraction procedures described below followed the manufacturer's instructions.

2.8.1 Extraction of DNA from whole blood

Two hundred microliters of whole blood were added to a microcentrifuge tube containing 200 μ L of lysis buffer (provided in the kit). The sample was mixed and 200 μ L of bead solution was added, and mixed by pulse vortexing for 15s and incubated for 10 min at room temperature. 200 μ L of ethanol (100 %) were added and mixed by pulse vortexing for 15s. The mixture was added to a QIAamp spin column and centrifuged for 1 min at 8000 rpm. The filtrate was discarded and the column was washed with 200 μ L of wash buffer AW1, by centrifuging for 1 min at 8000 rpm, followed by 200 μ L of wash buffer AW2 (14000 rpm, 3min). The filtrate was discarded and the column was washed with 200 μ L of lysis buffer AW3 in a fresh 1.5 ml microcentrifuge tube. The bound extracted DNA was eluted by adding 200 μ L of buffer AE and centrifuging for 1 min at 8000 rpm. The extracted DNA was stored at 4 $^{\circ}$ C or -20 $^{\circ}$ C until used for PCR.

2.8.2 Extraction of DNA from paraffin embedded tissue samples

2.8.2.1 Removing the paraffin by xylene

From each tissue, a block approximately 5 x 3 x 5 mm was finely dissected and washed three times with PBS. The extraction procedure followed the manufacturer's instruction (QIAamp DNA mini kit, Qiagen). A small section (not more

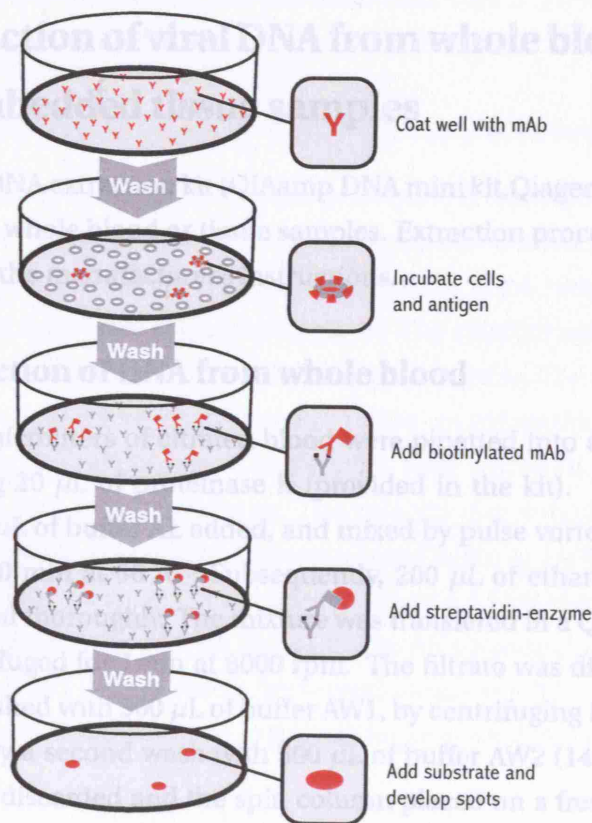


Figure 2.3: Principle steps of ELISpot assay.

2.7.4.1 Calculating the number of responder cells

The number of PBMCs responding to peptide stimulation was calculated as followed. The mean number of positive cells in the negative wells (without peptide, background) were deducted from mean number of spots in the positive wells (with peptide, triplicates) and expressed as percentage of PBMCs responding to peptide stimulation by interferon- γ secretion.

2.8 Extraction of viral DNA from whole blood or paraffin embedded tissue samples

A commercial DNA extraction kit (QIAamp DNA mini kit, Qiagen) was used to extract DNA from whole blood or tissue samples. Extraction procedures described below followed the manufacturer's instructions.

2.8.1 Extraction of DNA from whole blood

Two hundred microliters of citrated blood were pipetted into a microcentrifuge tube containing 20 μ L of proteinase K (provided in the kit). The sample was mixed and 200 μ L of buffer AL added, and mixed by pulse vortexing for 15s and incubated for 10 min at 56 °C . Subsequently, 200 μ L of ethanol (100 %) were added and mixed thoroughly. The mixture was transferred in a QIAamp spin column and centrifuged for 1min at 8000 rpm. The filtrate was discarded and the column was washed with 500 μ L of buffer AW1, by centrifuging for 1 min at 8000 rpm, followed by a second wash with 500 μ L of buffer AW2 (14000 rpm, 3min). The filtrate was discarded and the spin column placed on a fresh 1.5 ml microcentrifuge tube. The bound extracted DNA was eluted by adding 200 μ L of buffer AE and centrifuging for 1 min at 8000 rpm. The filtrate, containing the extracted DNA was stored at 4 °C or -20 °C until used for PCR.

2.8.2 Extraction of DNA from paraffin embedded tissue samples

2.8.2.1 Removing the paraffin by xylene

From each tissue, a block approximately 5 x 5 x 5 mm was finely dissected and washed three times with PBS. The extraction procedure followed the manufacturer's instruction (QIAamp DNA mini kit, Qiagen) A small section (not more

than 25 mg) of paraffin embedded tissue was placed in a 2 ml microcentrifuge tube and 1.2 ml of xylene was added. The tube was vigorously vortexed, followed by a centrifugation step in a microcentrifuge (13000 rpm, 5 min at room temperature). The supernatant was carefully removed and 1.2 ml ethanol added to the pellet to remove any residual xylene (vortexed gently). The tube was centrifuged again (5 min, 13000 rpm) and the supernatant removed. Twelve hundred microliters of ethanol was added, mixed and centrifuged again. The tube was left open for 15 min after the supernatant has been removed, which allowed the ethanol to evaporate. The tissue pellet was resuspended in 180 μ L ATL buffer (Qiagen).

2.8.2.2 Extraction of DNA from tissue with QIAamp DNA mini kit

To the tissue (see section 2.8.2.1) 20 μ L of proteinase K was added and incubated at 56 °C until the tissue completely lysed. Three hundred microliters of buffer AL were added, mixed by vortexing and incubated at 70 °C for 10min. Two hundred microliters of ethanol were added and mixed. The mixture was transferred to a QIAamp spin column placed on a 2 ml centrifugation tube. The spin column was placed on a new 2 ml micro-centrifuge tube after centrifugation at 8000 rpm for 1 min. Five hundred microliters of buffer AW1 were added to the spin column and centrifuged for 1 min at 8000 rpm. The spin column was placed on a new 2 ml tube, 500 μ L buffer AW2 added and centrifuged again at 14000 rpm for 3 min. After centrifugation, the spin column was placed on a 1.5 ml microcentrifugation tube and the column eluted with 200 μ L buffer AE by centrifuging at 8000 rpm for 1 min. The eluted DNA is stable for several weeks at 4 °C or longer at -20 °C. One microgram of DNA was used for all subsequent analyses (see section 2.9.1 and 2.9.2).

2.9 Detection of HCMV by PCR

2.9.1 Qualitative HCMV PCR

Qualitative HCMV PCR was performed based on a method described in [76]. Five microliters of DNA, extracted from whole blood (see section 2.8) were added to 100 μ L PCR reaction mix, containing 25 mM Tris pH 8.4, 17 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 10 mM 2-mercaptoethanol, 0.002 % (w/v) gelatin, 200 μ M final concentration of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP),

100 ng of each gB1 and gB2 primer (see table 2.1) and 1 unit Taq polymerase. Individual PCR reactions were performed in 0.5 mL microcentrifuge tubes (100 μ L) with 120 μ L mineral oil overlay. The thermal cycling condition were 39 cycles at 94 °C , 60 °C and 72 °C for 30 s each with a final cycle of 10 min at 72°C . The PCR reaction was performed in a Hybaid TR2 thermal cycler (Hybaid). HCMV specific DNA amplicons were visualized following agarose gel electrophoresis (3 % (w/v)) containing 40 ng/mL ethidium bromide. The sensitivity of the PCR was 200 genomes/ml. It has been shown that the primers are detecting all HCMV strains [237, 45].

2.9.2 Quantitative competitive HCMV PCR (QC-PCR)

Quantitative competitive HCMV PCR was carried out as described in section 2.9.1 with the following modification [76]. Each PCR reaction was spiked with a control sequence, identical in length and sequence to the amplified sequence with the exception of 2 bp. This change in the sequence was introduced with site directed mutagenesis, so generating a new *Hpa I* restriction site, which enables the distinction between wild type HCMV sequence from control sequence after endonuclease digest with the restriction enzyme *Hpa I*. After PCR amplification according the conditions in 2.9.1 PCR sample was digested overnight with 1 unit *Hpa I* and seperated on a 12% polyacrylamide gel. The polyacrylamide gel was stained with ethidium bromide (0.15 μ g / ml) for 5 min. HCMV virus load was calculated after scanning the stained gel on a flat-bed scanner (200 dpi resolution). The calculation was based on comparison of the intensity of the target sequence with the intensity of the control sequence of known copy number.

2.9.3 Quantitative TaqMan HCMV PCR

2.9.3.1 Principle of the TaqMan assay

The TaqMan assay [115] is based on two findings. First, the *Taq* polymerase exhibits a 5' to 3' exonuclease activity [120] and the concept of fluorescence resonance energy transfer (FRET) [275, 30]. The TaqMan assay is now commercially available together with an semi automated PCR machine from ABI (ABI 7700, ABI) and the appropriate software. In the TaqMan assay, the Taq polymerase enzyme cleaves an internal labeled nonextendable probe (the TaqMan probe) during the extension phase of the PCR. The TaqMan probe is labeled at the 5'-end

with the reporter dye 6-carboxyfluorescein (FAM) and with a quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. Because of the proximity of the two dyes in the intact TaqMan probe, the emission of the reporter dye is absorbed by the quencher dye. During the extension phase, the Taq polymerase degrades the internal TaqMan probe from the 5'-end and reported and quencher dye become separated. The reported dye transmission is no longer transferred to the quencher dye (no FRET), which results in a fluorescence emission at 518 nm when FAM is the reporter dye. The emission increases with every PCR cycle and can be read by a photomultiplier. A computer program calculates the increase of emission over the PCR cycles (R_n). The computer program constructs an amplification plot (see figure 2.4). An arbitrary threshold is set and for each PCR reaction, the cycle number is calculated when R_n exceeds the threshold. This point is called C_t . Because the C_t decreases linearly with increasing input quantity, the C_t value can be used to quantify the genome copy number in the sample (figure 2.4).

Table 2.1: HCMV, HHV-6 and HHV-7 specific PCR primers

Virus	Primer name	Sequence 5' → 3'
HCMV	gB1	GAGGACAACGAAATCCTGTTGGGCA
HCMV	gB2	GTCGACGGTGGAGATACTGCTGAGG
HCMV	gB3	CAATCATGCGTTTGAAGAGGTAGTCCACG
HHV-6	U67a	AAGCTTGCACAATGCCAAAAAACAG
HHV-6	U67b	CTCGAGTATGCCGAGACCCCTAATC
HHV-6	U67c	TCCATTATTTTGGCCGCATTCGT
HHV-6	U67d	TGTTAGGATATAACCGATGTGCGT
HHV-7	U42p1	TTTTTACATTTGGCTTGCTTTTTG
HHV-7	U42p2	ATATTTCTGTACCTATCTTCCCAA
HHV-7	U42p3	TGCTTTTTGGTTTGTAATTC
HHV-7	U42P4	GAATTTATGGAGTTTGGTCTG

2.9.3.2 TaqMan HCMV PCR

Real time amplification of HCMV DNA used glycoprotein B (gB or gp116) specific primers as previously described (76), with the forward primer sequence (gB1) and the reverse primer sequence (gB2), corresponding to nucleotides 1942 to 1964 and 2056 to 2071 of the HCMV gB ORF. The 130 bp product was detected in

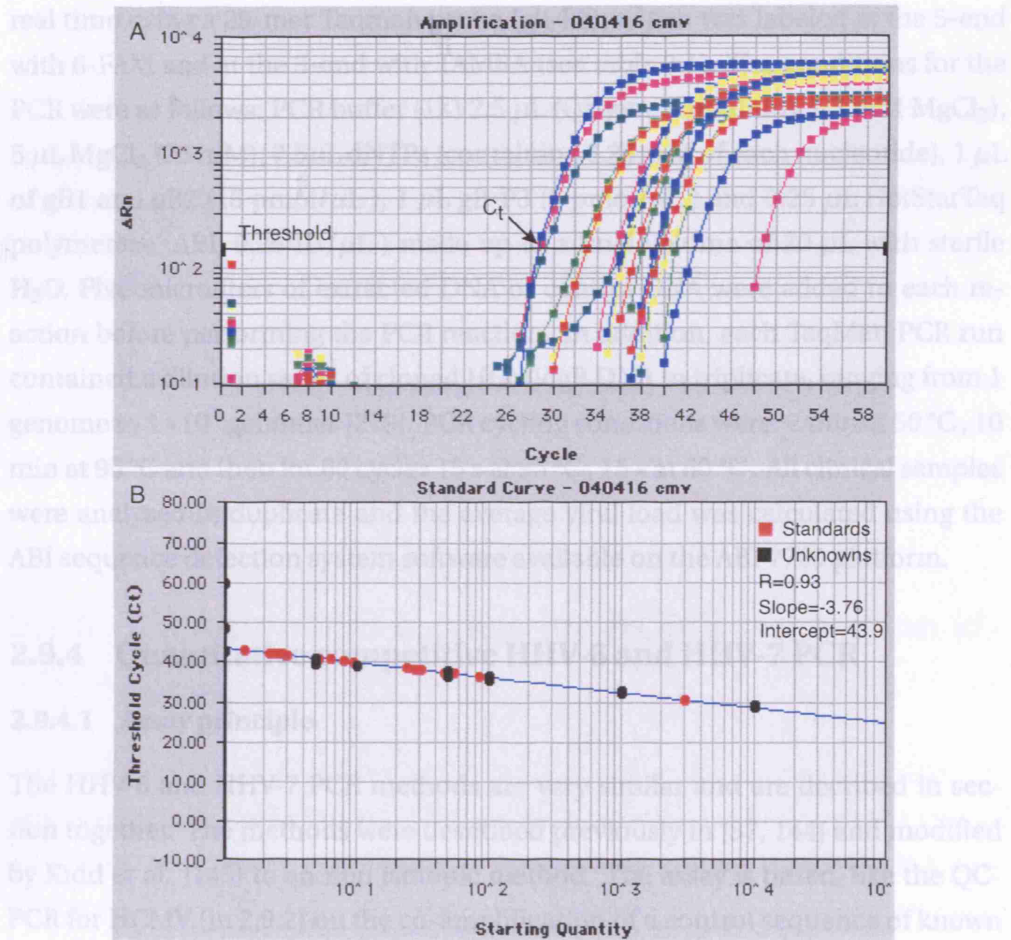


Figure 2.4: Top panel showing a typical amplification curves. Lower panel shows the calculated viral load standard curve together with viral loads of clinical blood samples

2.9.3.2 TaqMan HCMV PCR

Real time amplification of HCMV DNA used glycoprotein B (gB or gpU₅₅) specific primers as previously described [76], with the forward primer sequence (gB1) and the reverse primer sequence (gB2), corresponding to nucleotides 1942 to 1964 and 2066 to 2031 of the HCMV gB ORF. The 150 bp product was detected in real time using a 29-mer Taqman probe (gb-P3), which was labeled at the 5-end with 6-FAM and at the 3-end with TAMRA (see table 2.1). The conditions for the PCR were as follows: PCR buffer (1X) 2.5 μ L (Qiagen, containing 1.5 mM MgCl₂), 5 μ L MgCl₂ (25 mM), 7.5 μ L dNTPs (containing 6.25 mM of each nucleotide), 1 μ L of gB1 and gB2 (15 pmol/ μ L), 1 μ L gb-P3 (5 pmol/ μ L) and 0.25 μ L HotStarTaq polymerase (ABI, 0.25 IU/ μ L) made up to a final volume of 20 μ L with sterile H₂O. Five microliters of extracted DNA or control DNA were added to each reaction before performing the PCR reaction. In addition, each TaqMan PCR run contained a dilution series of cloned HCMV gB DNA in triplicate, ranging from 1 genome to 1×10^4 genomes [279]. PCR cycling conditions were: 2 min at 50 °C, 10 min at 95 °C and then for 60 cycles 15 s at 95 °C, 15 s at 60 °C. All clinical samples were analysed in duplicate and the average viral load was calculated using the ABI sequence detection system software available on the ABI 7700 platform.

2.9.4 Quantitative competitive HHV-6 and HHV-7 PCR

2.9.4.1 Assay principle

The HHV-6 and HHV-7 PCR methods are very similar and are described in section together. The methods were described previously in [37, 144] and modified by Kidd et al. [145] to a non isotopic method. The assay is based, like the QC-PCR for HCMV (in 2.9.2) on the co-amplification of a control sequence of known copy number in the same reaction. The control sequence is identical to wild type sequence, but was modified by the introduction of a new restriction endonuclease site (Sma I) in the middle of the sequence allowing discriminating wild type amplicon from control amplicon after polyacrylamide gel electrophoresis. In contrast to the HCMV PCR the HHV-6 and 7 PCR is nested. After the first round of amplification (primer 6a, and 6b for HHV-6, p1 and p2 for HHV-7), a second primer set, annealing within the amplicon generated during the first round (primer 6c and 6d, HHV-6; p3 and p4, HHV-7, see 2.1, 84,).

2.9.4.2 PCR conditions

The PCR was carried out in a total volume of 50 μL , containing 100 ng of each primers, 2mM MgCl_2 , 200 μM of each dNTP in 1 \times PCR buffer, containing 1 unit Taq polymerase and test specimen. To each sample 2 μL of control sequence was added with a defined copy number (1, 10, 100, 1000 and 10000 copies per 2 μL). PCR cycling condition were as follows: 95 $^\circ\text{C}$ 6 min, 95 $^\circ\text{C}$, 50 $^\circ\text{C}$, 72 $^\circ\text{C}$ each for 30 s (39 cycles), followed by one cycle with 10 min extension (72 $^\circ\text{C}$). One μL from the first round was added to a new reaction tube containing the inner primer set in addition to 1 \times PCR buffer, MgCl_2 , dNTPs and Taq polymerase (1U) with the following cycling conditions: 95 $^\circ\text{C}$ for 6 min, 95 $^\circ\text{C}$ 30 s, 50 $^\circ\text{C}$ for 30 s for HHV-6 and 60 $^\circ\text{C}$ for 30 s for HHV-7, 72 $^\circ\text{C}$ 30s; 17 cycles followed by one cycle with a final extension at 72 $^\circ\text{C}$ for 10 min. Ten microliters of the PCR products were taken into fresh reaction tube with 1.5 μL of 10x *Sma I* reaction buffer and 4.5 μL of *Sma I*. The digest was incubated for at least 3 h at 25 $^\circ\text{C}$ and analysed on a 12 /% non denaturing polyacrylamide gel, stained with ethidium bromide and scanned on a flat bed scanner into a computer readable format. Copy numbers were calculated with a gel image software (NIH image, NIH Bethesda).

2.10 Mathematical models for HCMV replication kinetics

The basic virus model is shown in figure 2.5 and described in detail in [188]. In this basic model, free virus (v) infects uninfected cells (x) at a constant rate β , which become infected (y) and release mature infectious virus with a rate k . Uninfected cells and infected cells die at a rate d and a . Free virus particles are eliminated with a rate u and uninfected cells are reproduced with a rate λ . This model can be described with a set of nonlinear differential equations:

$$\dot{x} = \lambda - dx - \beta xv \quad (2.1)$$

$$\dot{y} = \beta xv - ay \quad (2.2)$$

$$\dot{v} = ky - uv \quad (2.3)$$

2.10.1 Calculation of replication and decline rate (ρ and κ)

The model can be simplified under the assumption that before infection $y = 0$ and $v = 0$ (t_0). If the basic reproductive ratio (R_0) is ≥ 1 , then the number of free viruses can be calculated with

$$v(t) = v_0 e^{\rho t} \quad (2.4)$$

and the replication rate can be calculated after solving the equation 2.4 to

$$\rho = \frac{\ln \Delta}{\Delta t} \quad (2.5)$$

2.10.2 Calculating the replication rate on serial virus load data

Serial log transformed virus load data obtained before initiating antiviral therapy were plotted and a linear curve fit performed on all data points (proFit 5.6, Quantumsoft). The obtained parameters were used to calculate two virus loads, and the replication rate (ρ) calculated with equation 2.5.

2.10.3 Calculation of decline rate (κ)

Under the assumption of a 100% effective antiviral drug $\beta=0$ and the basic viral model in figure 2.5 can be described with:

$$\dot{y} = -av \quad (2.6)$$

$$\dot{v} = ky - uv \quad (2.7)$$

Solving these differential equations lead to:

$$y(t) = y e^{-at} \quad (2.8)$$

$$v(t) = \frac{v(ue^{-at} - ae^{at})}{u - a} \quad (2.9)$$

Virus load declines exponentially:

$$v(t) = v e^{-at} \quad (2.10)$$

after an initial shoulder phase.

2.10.4 Calculating the decline rate on serial virus load data

The decline rate (κ) was calculated in the same way as the replication rate, described in section 2.10.2.

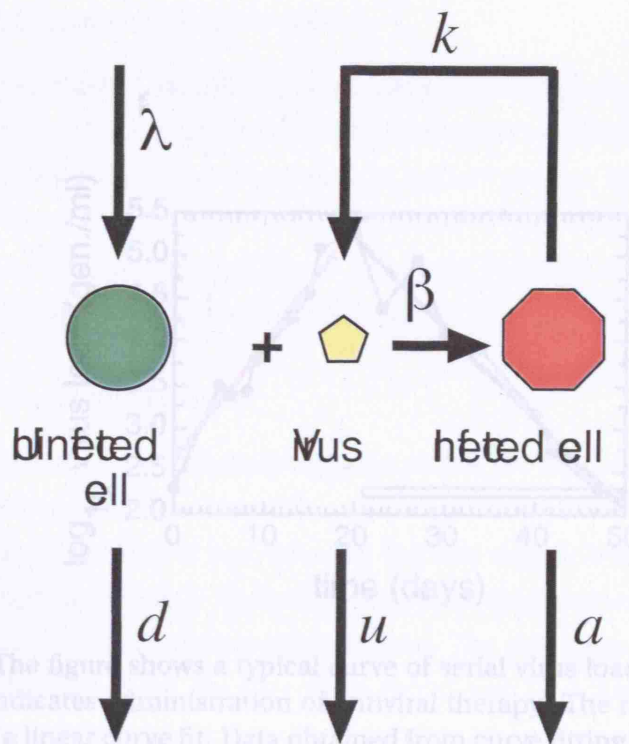


Figure 2.5: Basic model of virus dynamics. Free virus infects uninfected cells with a constant rate β , which produce free virus particle with a constant rate k . Uninfected cells, free virus and infected cells die or are eliminated with rates d , u and a . Fresh uninfected cells are reproduced with a rate λ (adapted from [188]).

2.11 Statistical methods

2.11.1 Comparison of continuous variables

Inferential statistics are used to assess if the observed difference between two groups happened by chance or if the difference reflects a true difference between the two groups (eg different medical treatment).

2.11.1.1 Unpaired two sample t-test

The t-test is applied, if samples were numerical in nature, random, and come from two unrelated normally distributed populations with equal variances. The test considers the difference in the means of the two groups. The null hypothesis (H_0) states, that the difference of the means is zero. In addition to the specific test statistic, a confidence interval can be calculated.

2.11.1.2 Mann-Whitney U-test

Numerical data from two unrelated groups which are not normally distributed can be compared. The Mann-Whitney U test is a nonparametric test and is equivalent to the two sample t-test, which is used on normally distributed data. The test assumes that two variables are independent and have a continuous distribution. The null hypothesis (H_0) states,

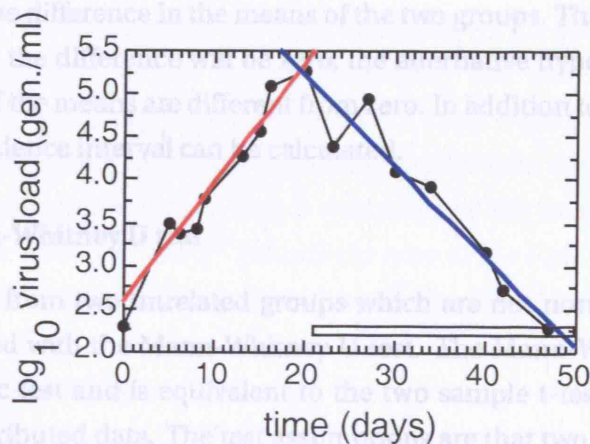


Figure 2.6: The figure shows a typical curve of serial virus load data. The horizontal bar indicates administration of antiviral therapy. The red and blue line, the result of a linear curve fit. Data obtained from curve fitting were used to calculate viral replication rate (ρ) and decline rate (κ).

the number of cases, the sum of the ranks of those cases, and the mean rank of those cases are calculated and a U-statistic is performed.

2.11.1.3 One way analysis of variance (ANOVA)

Samples from a number of independent groups (more than two) are compared with one way analysis of variance. The one way analysis of variance null hypothesis (H_0) states that all means of the groups are equal, the alternative hypothesis (H_1) that the means are unequal. The ANOVA assumes that the groups are defined by levels of a single factor, and that the variable of interest is normally distributed with an equal variance in each group. The analysis separates the total

2.11 Statistical methods

2.11.1 Comparison of continuous variables

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2.11.1.1 Unpaired two sample t-test

The t-test is applied, if samples were numerical in nature, random, and come from two unrelated normally distributed populations with equal variances. The test considers the difference in the means of the two groups. The null hypothesis (H_0) states, that the difference will be zero, the alternative hypotheses (H_1) that the difference of the means are different from zero. In addition to the specific test statistic, a confidence interval can be calculated.

2.11.1.2 Mann-Whitney U test

Numerical data from two unrelated groups which are not normally distributed can be compared with the Mann-Whitney U test. The Mann-Whitney U test is a nonparametric test and is equivalent to the two sample t-test, which is used on normally distributed data. The test assumptions are that two variables are independent and have a continuous distribution. The null hypothesis (H_0) states, that the medians are equal, the alternative hypotheses (H_1) that the medians are different. Mann-Whitney U test pools all the data and computes a rank for each value in the combined set. For each variable, the number of cases, the sum of the ranks of those cases, and the mean rank of those cases are calculated and a U-statistic is performed.

2.11.1.3 One way analysis of variance (ANOVA)

Samples from a number of independent groups (more than two) are compared with one way analysis of variance. The one way analysis of variance null hypothesis (H_0) states that all means of the groups are equal, the alternative hypotheses (H_1) that the means are unequal. The ANOVA assumes that the groups are defined by levels of a single factor, and that the variable of interest is normally distributed with an equal variance in each group. The analysis separates the total

variability in the data into the variability which comes from each group and the variability within each group.

2.11.2 Independence of categorical data

The Chi-square test and Fisher's exact test are used to test the hypothesis if the data in the groups are independent from each other.

2.11.2.1 χ^2 -squared test

The null hypothesis associated with the chi-square (χ^2) test states that two factors are statistically independent (H_0). The probability that a random case falls in a specific cell of a contingency table depends only on the probability that the case falls in a specified column and the probability that of a specific row. For each cell in the contingency table the number of cases expected is calculated if the null hypothesis is true. If the null hypothesis is false than the observed counts differ from the expected values, which is described by the standardized residuals calculated for each cell. The χ^2 value is the sum of the squared standardized residuals. If the χ^2 is relatively large then the null hypothesis is rejected.

2.11.2.2 Fisher's exact test

The Fisher's exact test is only used used for 2 x 2 contingency tables and if the numbers in the contingency table are small. The Fisher's exact test considers all possible 2 x 2 tables with the same marginal frequencies as observed in the table. The test then computes the probability of obtaining a table with the same marginal frequencies for which the association would be stronger than the association in the observed table. This value lies within the interval of 0 to 1, where 0 means the strongest possible association and 1 no association.

2.11.3 Linear regression analysis

The linear regression analysis investigates the relationship between two continuous numerical data, where one variable is dependent from the other (the predictor or explanatory variable). The simple linear relation can be described by:

$$Y = a + bx \tag{2.11}$$

where a as the intercept of the estimated line and b is the slope of the line. The parameter a and b are calculated by the method of least squares, which is minimizing the residuals (the distance of the observed datapoints from the calculated line). The linear regression is analyzed using the analysis of variance table. This table gives information about how much Y can be explained by the predictor (expressed as percentage and denoted by R^2) and how much by the residual error.

2.11.4 Univariable logistic regression

The logistic regression model extends the concept of the linear regression to a binary dependent variable. In contrast to the continuous dependent variable, in the simple regression, the response variable in the logistic regression model has only two values, yes or no (0 or 1). Because the response variable has only two states, the mathematical equation of the linear regression is transformed with the logit transformation:

$$\text{logit}(p) = \ln \frac{P}{1-P} \quad (2.12)$$

The logit transformation transforms probabilities into odds. The logistic regression can then be written as:

$$\text{logit}(P) = \ln \frac{P}{1-P} = \beta_0 + \beta_1 x \quad (2.13)$$

with x as explanatory variable, a the constant term, b the estimated logistic regression coefficients and P the predicted value of the logit(p).

After computing β_0 and β_1 , the equation

$$P = \frac{e^{\beta_0 + \beta_1 x}}{1 + e^{\beta_0 + \beta_1 x}} \quad (2.14)$$

can be used to obtain the probabilities of the logistic regression.

2.11.5 Multivariable logistic regression model

Logistic regression can be extended to multiple predictor variables and an extension to the logistic regression described in 2.11.4. The general multiple regression model for n predictors is:

$$\text{logit}(P) = \beta_0 + \beta_1 x_1 + \dots + \beta_n x_n \quad (2.15)$$

with the intercept β_0 , β_1 to β_n the partial regression coefficient for x_1 to x_n . Each partial regression coefficient is estimated by holding the remaining constant. Statistical software packages produce a table containing the parameter estimate (β), the standard error (ϵ), a p-value, odds-ratio and the 95% confidence interval (CI_{95%}) for each variable.

2.11.6 Survival analysis

The survival analysis addresses the question how long it takes for a patient to reach the endpoint of the study. In addition, the data are censored, meaning that all patients starting from a baseline (eg time of randomization) and the time is measured until a patient reaches a defined endpoint of the study (right censored data). The survival curves are calculated by the *Kaplan-Meier* method, which displays the cumulative probability of an individual being free of the endpoint at any time after the baseline. The *log rank test* is used to test for the hypothesis that there is no difference between the Kaplan-Meier curves.

2.11.7 Receiver operator curve

The receiver operating curve (ROC) helps to define a threshold of a biological test, which separates *no disease* from *disease* or to compare two different tests. With the ROC, a threshold is chosen which optimizes a test for either sensitivity or specificity. The ROC is a plot of sensitivity against 1-specificity, for different cut-off values. The overall ability of a test to discriminate between disease and no disease can be measured by the area under the ROC. A test with 100% sensitivity and specificity has a assigned value of 1.

2.11.7.1 The likelihood ratio

For any given datapoint in a ROC a likelihood ratio can be calculated with

$$likelihood\ ratio = \frac{sensitivity}{1 - specificity} \quad (2.16)$$

The threshold value which gives the highest likelihood ratio optimizes the test for disease. For example a likelihood ratio of 4, indicates that a patient with a greater value then the threshold is 4 times more likely to have the disease.

Chapter 3

Detection of owl's eye inclusions by histology

3.1 Introduction

Cytomegalovirus (HCMV) is an important cause of multiple organ dysfunction in the immunocompromised host [100]. Patients can present with hepatitis, pneumonitis, ulceration of the oesophagus or colon, retinitis, or encephalitis. Organ involvement is routinely diagnosed by biopsy, with visualisation of owl's eye intranuclear inclusions in stained tissue sections. [259, 169]

HCMV (human herpesvirus 5) is the prototype member of the *beta*-herpesvirinae, a subfamily of the herpesviridae [283]. In 1986 and 1990, respectively, two new herpesviruses were described and allocated to the *gamma*-virinae on the basis of their strong genetic relatedness to HCMV; these viruses are termed human herpesvirus 6 (HHV-6) [245] and HHV-7 [245, 15]. HHV-6 and HHV-7 can each cause febrile illness in young children, including exanthem subitum, [303, 277, 38] and case reports suggest that, like HCMV, HHV-6 may cause end organ disease in the immunocompromised host [151]. HHV-6 and HHV-7 infection are frequent in immunocompromised host especially in patients undergoing solid organ transplantation [146, 191, 96, 225]

Other reports suggest that HCMV associated disease might be increased in patients co-infected with HHV-7 [192, 146] or HHV-6 [51]. It is not known whether HHV-6 and/or HHV-7 can produce owl's eye inclusions in vivo but, if they do, this could complicate the interpretation of a postulated association between these other viruses and HCMV associated disease.

We have developed quantitative competitive polymerase chain reaction (QCPCR) methods to detect each of these three β -herpesviruses [76, 37, 144] and quantify the viral load in biological samples, including tissue specimens [61]. In our study, we used these techniques to determine the sensitivity of histopathological visualisation of owl's eye inclusions to detect HCMV infection and whether their presence is specific for HCMV alone among the β -herpesvirinae. In addition, this study determined if higher virus load in the tissue needed before owl's eye inclusion bodies are observed.

3.2 Materials and Methods

3.2.1 Clinical samples

To define the prevalence of HCMV infection in patients with AIDS we prospectively collected multiple tissues from all such patients undergoing necropsies at this institution. For these clinicopathological studies, we aimed to collect up to 14 organs from each necropsy (lymph node, spleen, brain, lung, heart, kidney, adrenal, oesophagus, duodenum, colon, pancreas, liver, stomach, and salivary gland). A total of 139 organs were available from 11 unselected human immunodeficiency virus (HIV) positive patients (median, 14 organs/patient; range, 9–14). The median CD4 count at death was 10/mm² (range, 0–20). Nine patients had been prescribed zidovudine during their illness but all died before protease inhibitor drugs became available [8].

3.2.2 Histopathological examination

The tissue samples were placed into buffered formalin during the course of a standard postmortem examination. After a minimum period of 48 hours in fixative, blocks were taken and processed through to paraffin wax. Sections were cut at 5 µm, stained with haematoxylin and eosin (Lillie's modification of Mayer's haematoxylin), and examined. The presence of typical owl's eye inclusions was recorded as a positive finding. If the morphological features were considered inconclusive, immunohistochemical staining (Dako monoclonal antibody; Dako, Cambridge, UK) was carried out to provide confirmatory evidence. All of the sections were read by a single independent pathologist observer (Dr JE McLaughlin, Consultant Pathologist, Royal Free Hospital).

3.2.3 Extraction of DNA from paraffin wax embedded tissue

From each tissue, a block approximately 5 × 5 × 5 mm was finely dissected and washed three times with sterile phosphate buffered saline. DNA was extracted from the tissue using the Promega Wizard DNA preparation kit (Promega, Southampton, UK), according to the manufacturer's instructions (section 2.8.2.1, page 80). The DNA was resuspended in water and 1 µg DNA used for all subsequent analyses (equivalent to 1.5 × 10⁵ diploid cells).

3.2.4 Methods for PCR and QCPCR for HCMV, HHV-6 and HHV-7

The methods used to detect HCMV, HHV-6, and HHV-7, both qualitatively and quantitatively, have been described in detail elsewhere [76, 37, 144] and in section 2.9.2 (page 82) and 2.9.4 (page 86). Briefly, the PCRs amplify genes UL55, U67, and U42 of HCMV, HHV-6, and HHV-7, respectively. The sensitivity of the methods was comparable, with the ultimate sensitivity of detection of HCMV being 5 genomes/ μg DNA, whereas the HHV-6 and HHV-7 QCPCR assays were capable of detecting 2 genomes/ μg DNA.

3.3 Results

Owl's eye inclusions were seen in 19 of 139 tissues (13.5%.) Inclusions were seen in organs from six of 11 patients. Inclusions were found on one or more occasion in 11 of 14 organs sampled (liver, stomach, and lymph node were negative in all cases).

Table 3.1,3.2 and 3.3 (page 101) shows the results of qualitative PCR testing. There was a significant association between the detection of HCMV by PCR and the presence of owl's eye inclusions ($p = 0.0004$).

No inclusions were seen in tissues that were HCMV PCR negative. In addition, 5 owl's eye positive tissue samples were negative for HHV-6 and HHV-7 DNA but positive for HCMV (one heart, kidney, pancreas, and two adrenals).

There was no association between the detection of HHV-6 and the presence of owl's eye inclusions, which were found in 13 of 100 (13 %) HHV-6 PCR positive tissues compared with six of 39 (15%) HHV-6 PCR negative samples. For HHV-7, there was a trend for inclusions to be found less frequently in tissues that were PCR positive for HHV-7 (nine of 92; 10%) compared with those that were HHV-7 PCR negative (10 of 47; 21%). This difference was of borderline significance ($p=0.07$).

3.3.1 Relationship between viral load and the presence of owl's eye inclusion bodies

We next analysed the relationship between viral load for HCMV, HHV-6, and HHV-7 in different organs and the visualisation of owl's eye inclusions in histological sections from these organs (Figure 3.1, page 102).

The HCMV viral load was significantly higher ($p < 0.001$; unpaired t-test) in samples positive for owl's eye inclusions (mean viral load, 5.35×10^6 genomes/ μ g DNA; range, $2 - 7.95 \times 10^6$ genomes/ μ g DNA), compared with samples where no owl's eye inclusions could be seen (mean viral load, 3.55×10^6 genomes/ μ g DNA; range, $1.3 - 5.99 \times 10^6$ genomes/ μ g DNA).

In contrast, no significant relation was found between the mean viral load for HHV-6 or HHV-7 from samples positive and negative for owl's eye inclusions. The mean viral load was slightly higher for HHV-6 (2.3×10^6 genomes/ μ g DNA; range,

Table 3.1: The number of organs that contained HCMV DNA related to the presence of owl's eye inclusions.

Owl's eye inclusions	HCMV DNA		
	Positive	Negative	Total
Yes	19	0	19
No	75	45	120
Total	94	45	139

$p < 0.001$

Table 3.2: The number of organs that contained HHV-6 DNA related to the presence of owl's eye inclusions.

Owl's eye inclusions	HHV6-DNA		
	Positive	Negative	Total
Yes	13	6	19
No	87	33	120
Total	100	39	139

$p = 0.78$

Table 3.3: The number of organs that contained HHV-7 DNA related to the presence of owl's eye inclusions.

Owl's eye inclusions	HHV7-DNA		
	Positive	Negative	Total
Yes	9	10	19
No	83	37	120
Total	92	47	139

$p = 0.07$

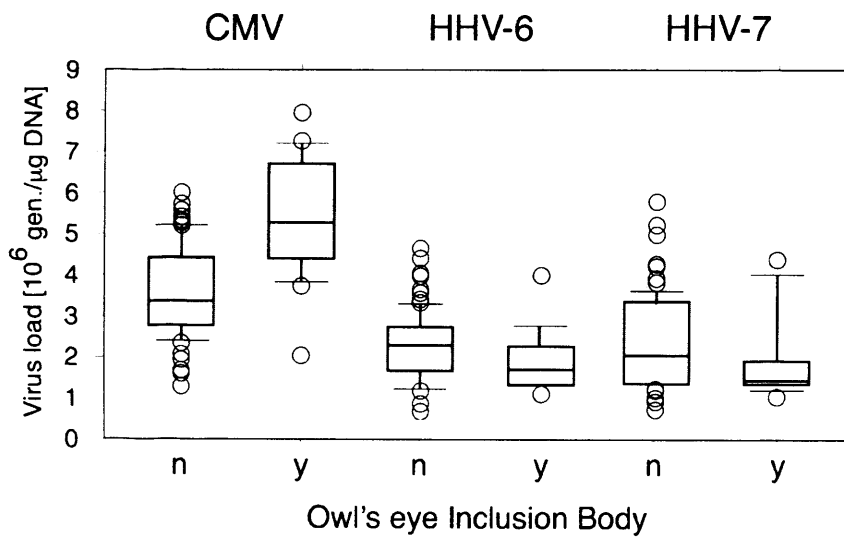


Figure 3.1: Box plots illustrating the relations between viral loads for the three β -herpesviruses and the presence of owl's eye inclusions. The horizontal lines display the 10th, 25th, 50th (thick line), 75th and 90th centiles; the boxes encompass 50% of the values; data points illustrating outlying values; n=no inclusions, y=inclusions.

0.7 – 4.6 × 10⁶ genomes/μg DNA) and HHV-7 (2.3 × 10⁶ genomes/μg DNA; range, 0.7 – 5.8 × 10⁶ genomes/μg DNA) in tissue samples negative for owl's eye inclusions, compared with samples positive for owl's eye inclusions (HHV-6 mean viral load, 1.9 × 10⁶ genomes/μg DNA; range, 1.1 – 3.9 × 10⁶ genomes/μg DNA; HHV-7 median viral load, 1.8 × 10⁶ genomes/μg DNA; range, 1 – 4.3 × 10⁶ genomes/μg DNA).

3.3.2 Quantitative relation between HCMV and owl's eye inclusion in different organs

Finally, we examined in detail the quantitative relation between HCMV, HHV-6 and HHV-7 virus load in organ tissue and the presence of inclusion bodies. (Figure 3.3.2, page 104; figure 3.3.2, page 105; figure 3.3.2, page 106). Although the numbers of individual organs were small, in general, owl's eye inclusions were seen in samples with higher HCMV viral loads, with the exception of lung tissues, whereas HHV-6 and HHV-7 virus load was not higher in organ tissue samples with owl's eye inclusion bodies.

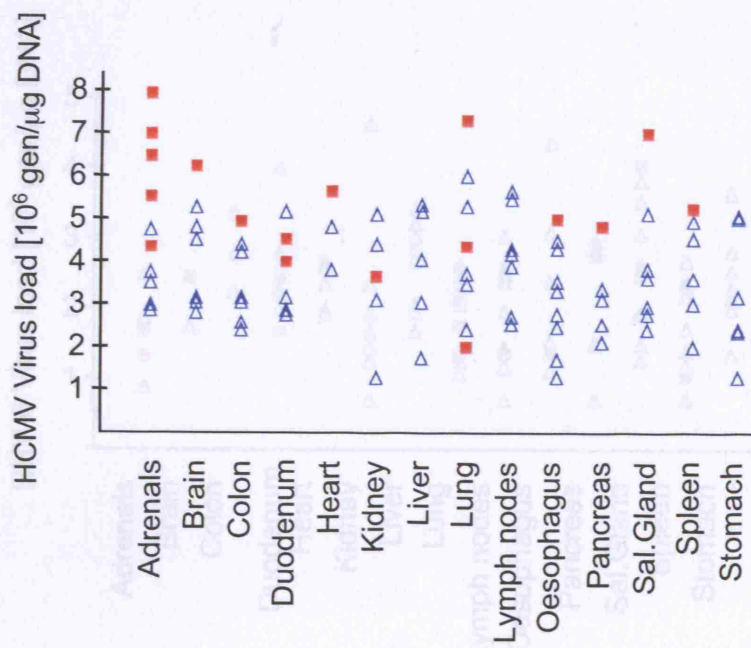


Figure 3.2: Association between the quantity of cytomegalovirus (HCMV) DNA and the presence of owl's eye inclusions. Red closed squares, HCMV load associated with owl's eye inclusions; open blue triangles, HCMV load associated with negative staining for owl's eye inclusions.

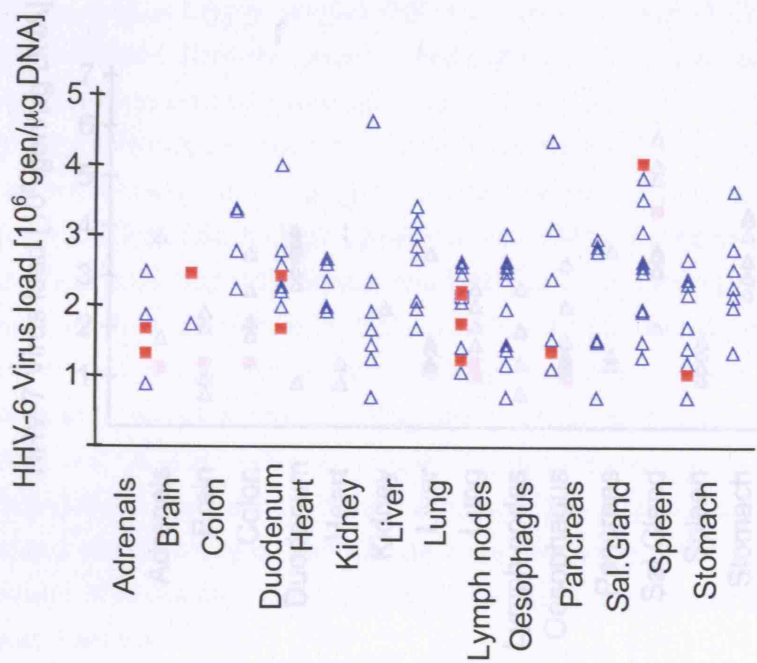


Figure 3.3: Association between the quantity of HHV-6 DNA and the presence of owl's eye inclusions. Red closed squares, HHV-6 virus load associated with owl's eye inclusions; blue squares, HHV-6 virus load associated with negative staining for owl's eye inclusions.

3.4 Discussion

The results of this investigation confirm the high specificity of owl's eye inclusions for the diagnosis of HCMV organ involvement. Specifically, the presence of inclusions correlated strongly with the detection of HCMV DNA by PCR and did not correlate with the detection of HHV-6 or HHV-7 DNA by PCR. We conclude that the more recently described members of the β -herpesvirinae either do not produce owl's eye inclusions that can be confused with those of HCMV, or that their incidence is so low as to make them undetectable by PCR. Other herpesviruses such as herpes simplex (HSV) or varicella zoster (VZV) can induce owl's eye inclusions. However, experienced histopathologists are able to differentiate between owl's eye inclusions induced by HCMV from HSV or VZV inclusion bodies.

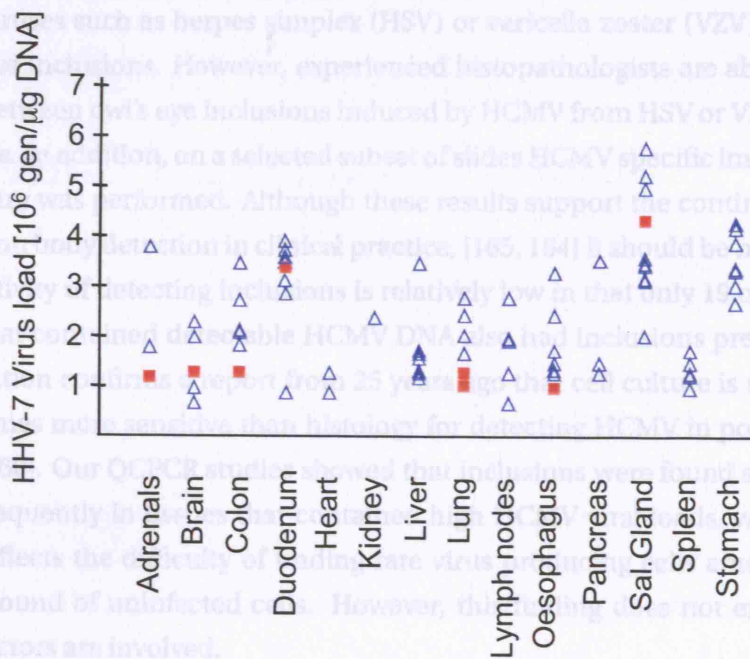


Figure 3.4: Association between the quantity of HHV-7 DNA and the presence of owl's eye inclusions. Red closed squares, HHV-7 virus load associated with owl's eye inclusions; open blue triangles, HHV-7 virus load associated with negative staining for owl's eye inclusions.

This work is important because it investigates the specificity of detecting owl's eye inclusions for the diagnosis of HCMV organ involvement. Specifically, the presence of inclusions correlated strongly with the detection of HCMV DNA by PCR and did not correlate with the detection of HHV-6 or HHV-7 DNA by PCR. We conclude that the more recently described members of the β -herpesvirinae either do not produce intranuclear inclusions that can be confused with those of HCMV, or that their incidence is so low as to make them undetectable by PCR. Other herpesviruses such as herpes simplex (HSV) or varicella zoster (VZV) can induce owl's eye inclusions. However, experienced histopathologists are able to differentiate between owl's eye inclusions induced by HCMV from HSV or VZV inclusion bodies. Our results show, although only 11 patients with different immunological background were investigated, that this is not the case and should facilitate future studies on the possible interactions between members of the β -herpesviruses in vivo.

3.4 Discussion

The results of this investigation confirm the high specificity of owl's eye inclusions for the diagnosis of HCMV organ involvement. Specifically, the presence of inclusions correlated strongly with the detection of HCMV DNA by PCR and did not correlate with the detection of HHV-6 or HHV-7 DNA by PCR. We conclude that the more recently described members of the β -herpesvirinae either do not produce owl's eye inclusions that can be confused with those of HCMV, or that their incidence is so low as to make them undetectable by PCR. Other herpes viruses such as herpes simplex (HSV) or varicella zoster (VZV) can induce owl's eye inclusions. However, experienced histopathologists are able to differentiate between owl's eye inclusions induced by HCMV from HSV or VZV inclusion bodies. In addition, on a selected subset of slides HCMV specific immunohistochemistry was performed. Although these results support the continued use of inclusion body detection in clinical practice, [165, 164] it should be noted that the sensitivity of detecting inclusions is relatively low in that only 19 of 94 (20%) organs that contained detectable HCMV DNA also had inclusions present. This observation confirms a report from 25 years ago that cell culture is approximately six times more sensitive than histology for detecting HCMV in postmortem tissues [260]. Our QCPCR studies showed that inclusions were found significantly more frequently in tissues that contained high HCMV viral loads, which presumably reflects the difficulty of finding rare virus producing cells among a large background of uninfected cells. However, this finding does not exclude that other factors are involved.

This work is important because it investigates the specificity of detecting owl's eye inclusions, which is part of the internationally agreed case definition of HCMV disease [165, 164]. Recent reports suggest that HCMV disease is more common among patients co-infected with HHV-7 [192, 146] or HHV-6 [51] One possible explanation for these observations could have been that HHV-6 and HHV-7 might themselves produce intranuclear inclusions and so lead to a false association with HCMV disease. Our results show, although only 11 patients with different immunological background were investigated, that this is not the case and so should facilitate future studies on the possible interactions between members of the β -herpesviruses in vivo.

Chapter 4

Natural History Study of CD8+ T-Cell Mediated Immunity against HCMV in Renal Transplant Patients

4.1 Introduction

Historically, HCMV infection has been a significant clinical problem following organ transplantation and can lead to a range of overt clinical symptoms such as prolonged fever, hepatitis, gastrointestinal disease (termed direct effects) and also a number of indirect effects including organ rejection. In recent years, the development of prophylactic and pre-emptive therapeutic patient management strategies has significantly reduced mortality attributed to HCMV and impacted positively on morbidity. A variety of agents (valaciclovir, oral ganciclovir and most recently valganciclovir) have been shown in controlled clinical trials to be effective for prophylaxis of solid organ transplant recipients at high risk of HCMV disease, such as HCMV seronegative patients receiving an organ from a HCMV seropositive donor [87, 301, 180, 167]. Controlled trials of pre-emptive therapy initiated on the basis of detection of high levels of virus in blood by PCR or antigenemia assays have also been shown to minimise disease and provide rapid control of replication [229, 224]. In addition to the high risk patients mentioned above, seropositive are also at risk of HCMV reactivation and also re-infection if receiving an organ from a seropositive donor and form an important group to consider as a subset will be destined to experience high level HCMV replication. HCMV replication *in vivo* is highly dynamic with doubling times between 0.4 and 2 days. The basic reproductive number (R_0) for HCMV in immune naïve liver transplant recipients is ~ 15 which reduces to 2.4 in patients who are already HCMV seropositive. Several studies, including many from our group, have shown that high level HCMV replication, as detected by high viral loads in blood by quantitative virological assays, is a significant risk factor for HCMV disease [65].

T-cell immune control is a fundamental effector process that controls HCMV replication *in vivo*. The CD8 T-cell immune response to HCMV is dominated by responses to the tegument protein ppUL83 and to the immediate early protein pUL123 [300] (see also 1.8.2, page 59. Data from many studies using class I HLA tetramer reagents have shown that the human host devotes a high proportion of the total CD8 T-cell response to the control of HCMV averaging $> 1\%$ in healthy individuals and reaching levels of 10-50 % in acute infection in immunocompromised hosts. In renal transplant patients, CD4 T-cell help is also necessary to maintain control of replication. While we have a substantial amount of qualitative data relating to the CD8 T-cell immune control of HCMV after trans-

plantation there are relatively few studies addressing whether specific quantitative malfunction of these CD8 T-cells, in addition to their frequency and absolute number, is also a factor that contributes to the failure to suppress replication. Accordingly, I set out to investigate the functional capacity of HCMV specific T-cells in patients who did, or did not develop high level HCMV replication after renal transplantation. Our results show that patients who proceeded to experience DNA viremia have an HCMV CD8 T-cell population that is functionally impotent compared to patients who maintained suppression of HCMV replication to low levels.

4.2 Materials and Methods

4.2.1 Study population and study design

A prospective natural history study, investigating the role of HCMV specific CD8 T-cells in controlling HCMV virus replication post renal transplantation was carried out in a single centre (Royal Free Hospital, London, UK) between October 2000 and December 2001. In total, 25 renal transplant patients entered the study, with the following HLA class I types: HLA-A*0201 (n=14), HLA-B*0702 (n=5), HLA-B*0801 (n=7) and HLA-B*3501 (n=6), see table 4.2, page 127.

4.2.2 HCMV surveillance

Citrated blood was collected at least twice a week while inpatients and whenever attending the outpatient clinic, for HCMV PCR. Blood samples positive for HCMV by qualitative PCR were quantified by quantitative HCMV PCR (TaqMan method, see 2.9.3.2).

4.2.3 Immunosuppression

All patients received Prednisolone together with Cyclosporin or Tacrolimus alone or in combination with Sirolimus, Tacrolimus or Azathioprine. One patient received Tacrolimus with Basiliximab, a monoclonal antibody against the interleukin-2 (IL-2) receptor. Two patients Tacrolimus, Sirolimus and Mycophenolate Mofetil (MMF) together. Table 4.3 gives a more detailed summary of the immunosuppressive medication a patient received.

4.2.4 Antiviral prophylaxis

No patients received antiviral prophylaxis against cytomegalovirus.

4.2.5 Pre-emptive antiviral treatment

Patients with two consecutive positive HCMV PCR samples received pre-emptive anti-HCMV therapy with either iv Ganciclovir (5 mg/kg bd adjusted to creatinine clearance, n=7 patients) or iv Foscarnet plus iv Ganciclovir each half dose (n=3). Antiviral therapy was continued until two consecutive negative PCR samples were obtained.

4.2.6 Immunological monitoring

Preservative free Li-heparin blood (20 ml) was collected for isolation of peripheral blood mononuclear cells (PBMC) on a weekly basis or whenever attending the outpatient clinic. PBMCs were isolated on the same day and cryopreserved for immunological studies (see also 2.1 at page 66).

4.2.6.1 Peptide sequences

The following peptides were used in the tetrameric complexes and in the ELISpot assay.

HLA class I	Peptide sequence	Reference
A2	NLVPMVATV	[300]
B7	TPRVTGGGAM	[300, 142]

4.2.6.2 Tetrameric complex staining

Cryopreserved PBMC's were thawed (see 2.2) and stained with HCMV pp65 tetrameric complexes as described in 2.4.

4.2.6.3 Lymphocyte surface marker staining

Isolated PBMC's were stained for CD8 (PE) as described in 2.5

4.2.6.4 CD8 ELISpot assay

If available, isolated PBMC's from the same time point used for the tetrameric complex staining were used to perform an ELISpot an assay as described in 2.7

4.2.6.5 Detection of HCMV in blood by PCR and quantification

Qualitative HCMV PCR is described in 2.9.1 on page 81. Quantitative competitive HCMV PCR is described in 2.9.2 on page 82, and real time quantification by the TaqMan method in 2.9.3.2 on page 86.

4.2.6.6 Statistical analysis

The ppUL83 specific HCMV CD8+ T-cell frequency was measured as a percentage of CD8 cells or total lymphocytes. Multiple stainings on the same patient sample were expressed as a median of the ppUL83 specific HCMV CD8+ T-cell frequency. The relative ppUL83 specific CD8+ T-cell frequency was calculated as percentage of CD8+ T-cells or as percentage of lymphocytes for the comparison with the ELISpot data. Samples with less than 20000 total events within the lymphocyte gate were excluded from the analysis. The number of cells secreting IFN- γ , obtained in a ELISpot assay, was expressed as percentage of cells secreting IFN- γ relative to the total cells used in the assay. These percentages were log transformed to provide a normal distribution before performing further statistical tests. Data were stratified in patients who became viremic, with further stratification in samples taken before, during and after viremia. Samples from patients who did not reactivate HCMV during the study period were stratified into specimens taken before day 50 post transplantation and samples taken after day 50. An ANOVA approach was applied to compare the samples between the different strata. Mann-Whitney U test was used to compare the tetrameric complex to ELISpot ratio between viremic and non-viremic patients. A simple linear regression model was used to compare the frequency of HCMV specific tetrameric positive T-cells and cells responding in a IFN- γ assay. Logistic regression models, using viremia as a binary outcome was used to identify and quantify risk factors associated with becoming HCMV viremic post renal transplantation. If not otherwise indicated, all summarized numbers are expressed as median with the range in brackets. The Mann-Whitney U test was used as a non parametric test for non log transformed data.

4.3 Results

4.3.1 Demographic data of study cohort

A total of 25 (17 male, 8 female) patients entered the study. Median follow up time was 363 days (range 86 to 680 days) with 13 (7 to 22) blood samples per patient available for immunological studies. Age ranged from 19 to 67 years (median 42 years). Four patients were HCMV IgG negative at transplantation and received an organ from a HCMV seronegative donor. None of these became HCMV viremic. In contrast, the two seronegative patients who received a kidney from a HCMV seropositive donor became viremic (primary HCMV infection). Eight of the 19 HCMV seropositive patients, who received either a HCMV negative or positive kidney became viremic. These HCMV immune experienced patient group were studied in more detail to determine factors associated with recurrent high level HCMV replication. A more comprehensive summary of patient demographics is shown in table 4.1. The HLA class I distribution is shown in table 4.2 on page 127.

4.3.2 Age and control of HCMV replication

Patients who became HCMV PCR positive during the study period were significantly older (median 56 (22-67) vs 29 (19-59), $p < 0.001$; Mann-Whitney U test). A sub-analysis, restricted to patients with pre-existing immunity showed a similar result (median age 61 (range 22-67) in viremic patients vs 29 (range 22-67), $p < 0.001$). The boxplot in figure 4.1, page 117 illustrates this finding.

4.3.3 Virological Markers of Study Cohort

Ten patients (40%) had at least one episode of HCMV viremia, defined as two consecutive positive HCMV PCR samples, and required antiviral therapy. The median time to become HCMV PCR positive was 38 days (range: 15-69), 32 days (median) in the immune experienced group (range 15-51 days) and 4 and 69 days respectively in the two immune naïve patients. All viraemic patients received pre-emptive antiviral therapy (median 16 days, range 8-35 days) and were HCMV PCR negative on two consecutive PCR samples when therapy was discontinued. Five of the 8 viraemic patients in the immune experienced group were PCR negative within 14 days of therapy, whereas both patients with a primary HCMV infection required therapy for more than 14 days. Baseline HCMV virus load (defined

as virus load at start of antiviral therapy) was 3.9 log₁₀ genomes/ml (range 3.05 to 5 log₁₀ genomes/ml) and 0.7 log₁₀ higher in the primary HCMV infection group. A summary of virological findings is shown in table 4.4 on page 128.

4.3.4 Quantification of HCMV pp65 specific CD8+ T-cells with tetrameric complexes

4.3.4.1 HCMV pp65 specific CD8+ T-cell frequency for individual patients

Trellis plots are able to show more than one factor within a two dimensional graph (for example viraemia and HCMV serostatus). The trellis plot in figure 4.2 shows the proportion of CD8 T-cells which bound pp65 tetrameric complexes over the study period. The plot separates viraemic patients (upper panel) from non viraemic patients (lower panel), and HCMV serostatus (NN, NP or PP, PN). Neither of the patients with a primary HCMV infection mounted a significant immune response against the pp65 protein. In addition, all seronegative patients who received a kidney from a seronegative donor did not show a pp65 specific immune response.

4.4 Individual pp65 tetrameric complex frequencies over time in non viraemic and in viraemic patients are shown in figure 4.3 (page 119) and figure 4.4 (page 120) respectively. Only patient R8 (A2), R10 (B7), R18 (B35) and R22 (B7) showed a sustained frequency of 1% or more in non viraemic patients. In contrast, to the non-viraemic patients, 40% of the viraemic patients showed a frequency of more than 2% on at least one specimen. The pp65 specific frequency increased in patient R7 (B35), R19 (A2) and R33 (A2) to more than 5% post viraemia. Eight (80%) of the viraemic and 12 (80%) of the non-viraemic patients had a pp65 tetrameric complex frequency of greater or equal than 0.1% on at least one blood sample.

4.3.4.2 HCMV specific CD8+ T-cell frequencies in viraemic versus non viraemic patients

In HCMV seropositive recipients (HCMV serostatus D-R+ or D+R+) who controlled HCMV replication following transplantation the median ppUL83 specific CD8+ T-cell frequency was 0.28% (0.02-3.35) prior to day 50 and 0.37% (0.01-6.92) after day 50. In patients who failed to control HCMV replication the CD8+ T-cell frequency was elevated prior to viraemia (0.71% (0.29-3.65)) during viraemia (1.19% (0.27-5.2)) and following viraemia (0.5% (0.03-6.46)) (table 4.6, page 130).

A one way ANOVA analysis showed that viremic patients had a significantly higher ppUL83 specific CD8+ T-cell frequency than patients who controlled HCMV replication ($p=0.008$). (figure 4.5).

4.3.5 Enumeration of functional HCMV specific CD8+ T-cells in HCMV seropositive patients

In order to complement the data obtained using class I HLA tetramer reagents I performed ELISpot analyses to ascertain the frequency of CD8+ T-cells that were able to secrete IFN- γ in response to peptide stimulation. In HCMV seropositive patients who controlled HCMV replication, the median frequency of IFN- γ positive CD8+ T-cells (as a percentage of the total PBMC population) was 0.023% (0.005-1.08) before day 50 and 0.032% (0.005-4.66) after day 50. In patients who failed to control HCMV replication, a frequency of 0.027% (0.005-0.009) was observed before viremia, 0.082% (0.005-0.87) during viremia and 0.173% (0.005-0.82) after viremia . In contrast to the results obtained with the ppUL83 HCMV specific tetramers, there was no significant difference in the frequency of IFN- γ cells after peptide stimulation between patients who did or did not control HCMV replication (One-way ANOVA $p=0.35$), figure 4.6, page 122 and table 4.7 on page 130.

4.3.6 Functional capacity of ppUL83 specific CD8+ T-cells and failure to control HCMV replication

While there was a strong correlation between the frequency of ppUL83 specific IFN- γ secreting cells and cells identified using ppUL83 specific tetrameric complexes in patients who did or did not control HCMV viremia ($R^2 = 0.49$ viremic patients, $R^2 = 0.41$ non viremic patients, 4.7, page 123). I observed that in patients with high level HCMV replication there were significantly fewer cells able to secrete IFN- γ , compared to patients who remained HCMV PCR negative. This observation is conveniently summarised in the boxplot shown in figure 4.9 (page 125), where the ratio of the CD8+ T-cells identified using either the HLA tetramer reagent or the IFN- γ ELISpot are compared. Data of individual patients are shown in figure 4.8 (page 124). In these analyses a ratio of unity implies that all tetramer positive CD8 T-cell have the ability to secrete IFN- γ . In non viremic patients 80.9% (median) of the tetrameric positive cells were able to secrete IFN- γ , com-

pared to only 22.1% (median) in the viremic patients. This difference was highly significant ($p < 0.0001$, Mann-Whitney U test).

4.3.7 Factors associated with high level HCMV replication post renal transplantation

A series of univariable and bivariable logistic regression models were used to determine the relative importance of the risk factors identified in this study. Importantly, these models were based on samples taken before becoming HCMV PCR positive and included all patient samples irrespective of donor and recipient serostatus. Univariable models showed that an increase of 1% in ppUL83 specific CD8 T-cell frequency was associated with 2.15 fold increase in becoming viremic. A 1% decrease in the frequency of IFN- γ producing PBMCs is associated with a 3.03 fold increase of the probability of viremia. However, an increasing percentage of IFN- γ secreting cells within the ppUL83 specific CD8 T cell population is associated with a 6.6 fold decreased risk of HCMV viremia (see table 4.5).

The probability of a patient experiencing high level HCMV replication based upon the relative competence of their CD8+ T-cells is shown in Figure 4.3.7 (page 126). The graph was constructed based on the interferon- γ to tetramer ratio as predictor for HCMV viremia (see table 4.5, page 129). Based on this model, the probability to become HCMV viremic in blood is 1%, if all HCMV pp65 tetrameric positive CD8+ T-cells are able to secrete IFN- γ . However the probability increased to 42% if only 10% of the tetrameric positive cells are able to secrete IFN- γ .

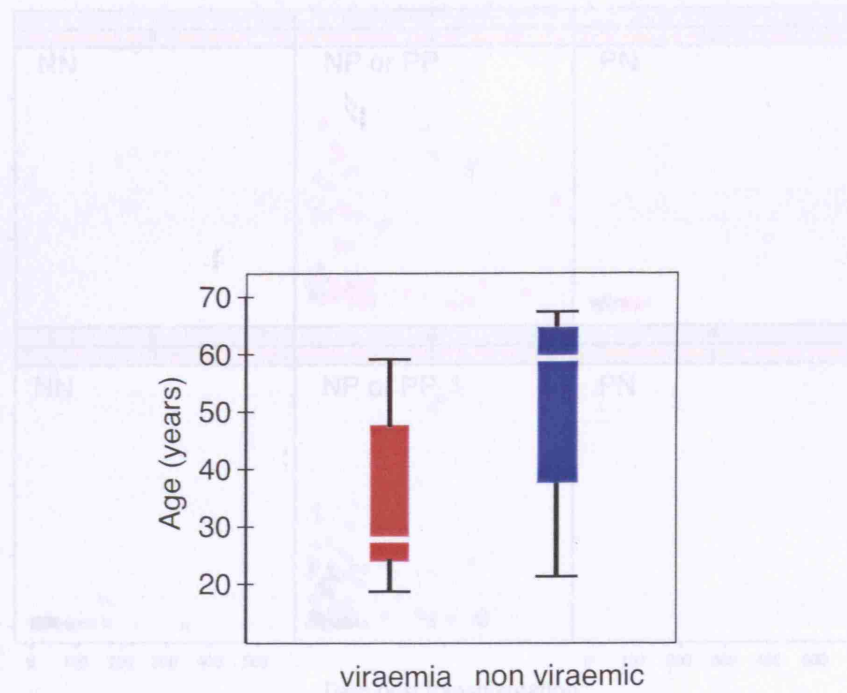


Figure 4.1: Boxplot showing the age distribution in patients with at least one episode of HCMV viraemia versus patients without viraemia.

patients (upper row) and non viraemic patients (lower row). In addition patients are grouped according the donor-recipient status. First column D-R- (NN); second column D-R+ (NP; or D-R+ (PP); third column D-R- (FN). Green, samples taken before becoming viraemic; red, samples taken during viraemic period; cyan, samples taken after the first episode of HCMV viraemia. The small numbers in the green field are descriptor for the viraemic status (0=no, 1=yes), the small numbers in the orange field a descriptor for the donor-recipient status.

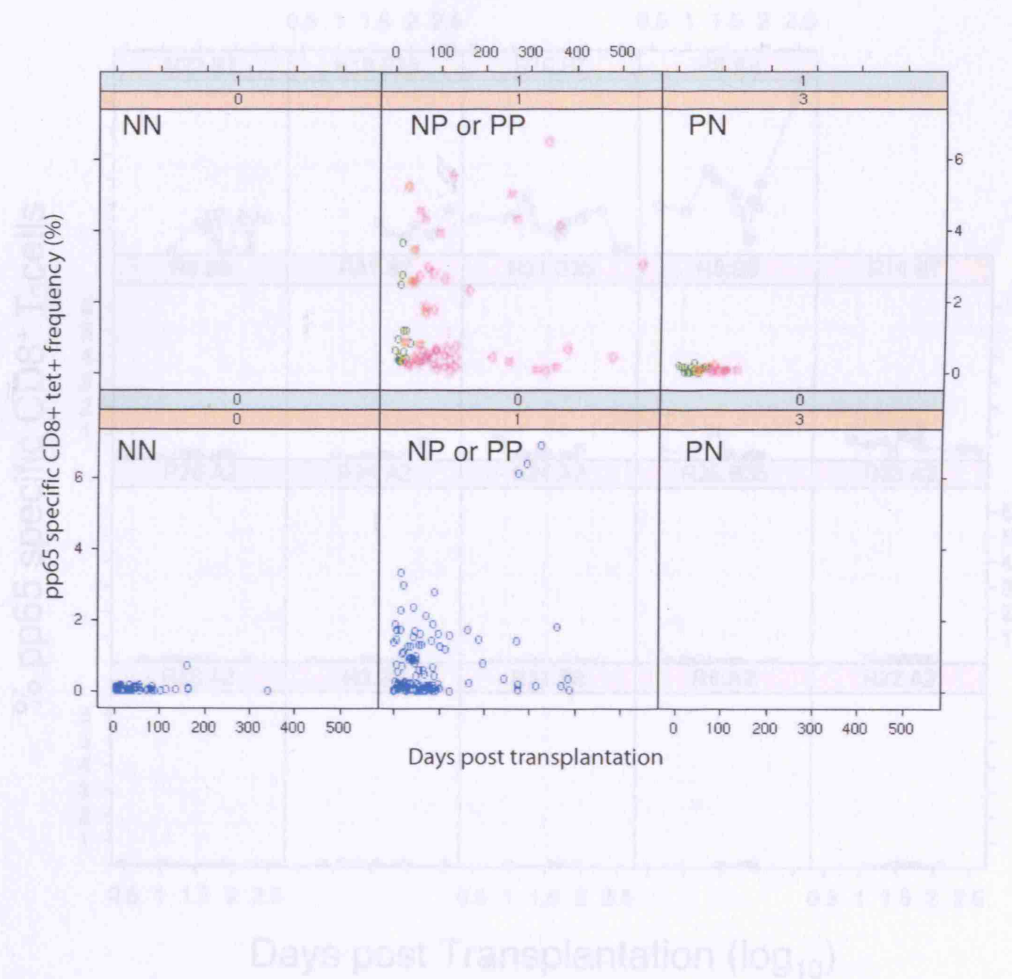


Figure 4.2: Trellis graph showing the frequency of ppUL83 (pp65) specific tetrameric complex positive CD8+ T-cells in viremic patients (upper row) and non viremic patients (lower row). In addition patients are grouped according the donor recipient status. First column D-R- (NN); second column D-R+ (NP) or D+R+ (PP); third column D+R- (PN). Green, samples taken before becoming viremic; red, samples taken during viremic period; cyan, samples taken after the first episode of HCMV viremia. The small numbers in the green field are descriptor for the viremic status (0=no, 1=yes), the small numbers in the orange field a descriptor for the donor-recipient status.

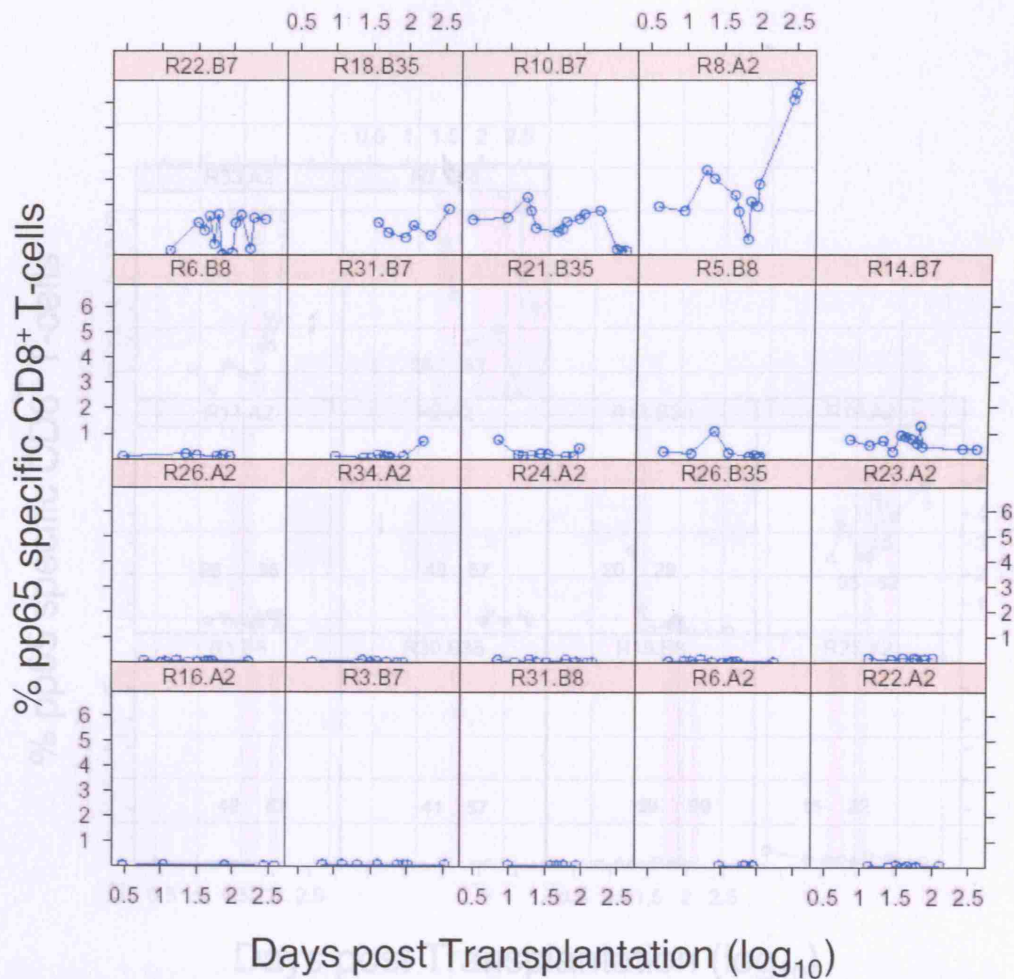


Figure 4.3: Longitudinal frequency of pp65 specific tetrameric complex positive CD8+ T-cells in viremic patients. Each box represents one patient. Vertical shaded bars indicate the viremic time period for each patient. To illustrate

Figure 4.3: Longitudinal frequency of pp65 specific tetrameric complex positive CD8+ T-cells in non viremic patients. Each box represents one patient. To illustrate the time course better, the x-axis is \log_{10} transformed.

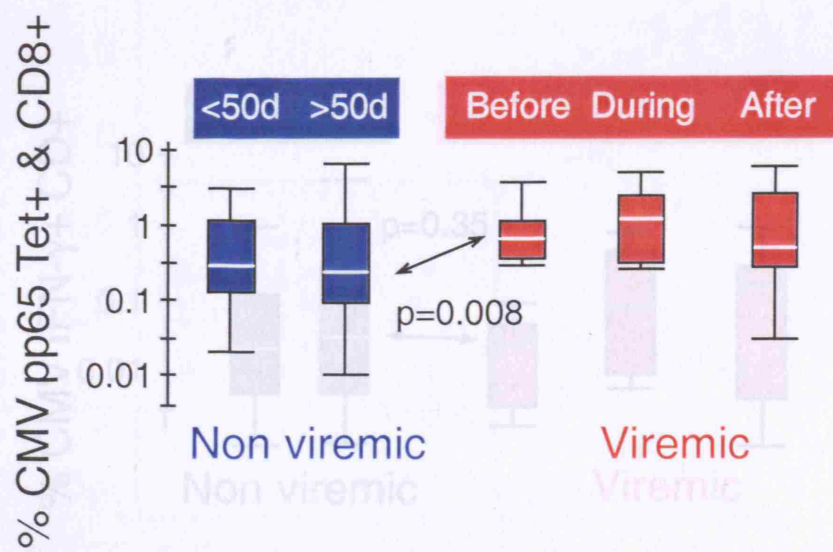


Figure 4.5: Boxplots showing the frequency of ppUL83 (pp65) specific tetrameric complex positive CD8+ T-cells in viremic and non viremic patients. Data from non-viremic patients are grouped according days post transplantation (< day 50; > day 50). Data from viremic patients grouped according the time relative to the occurrence of HCMV viremia.

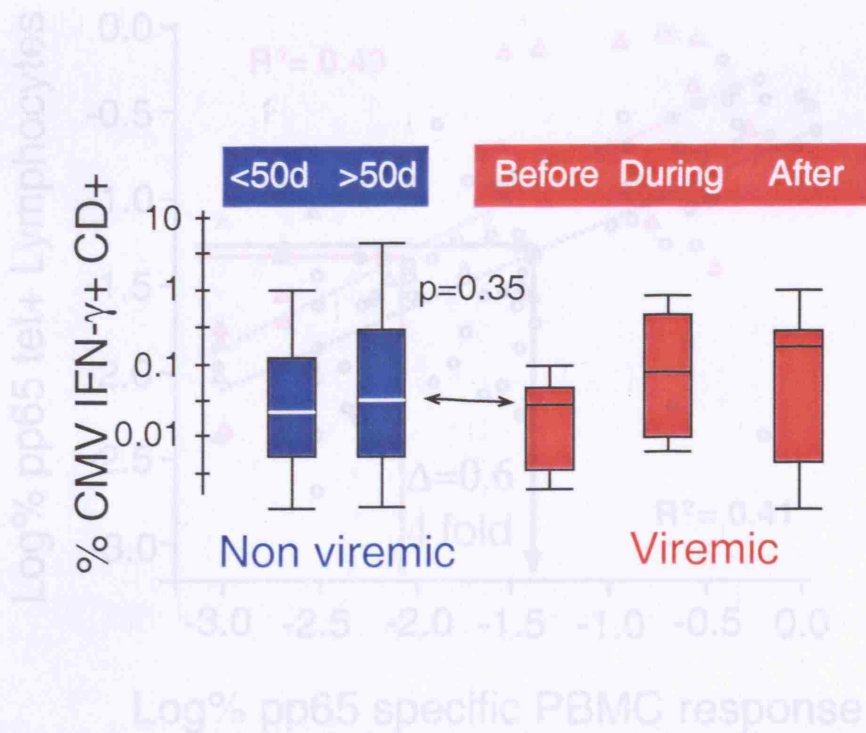


Figure 4.6: Boxplot illustrating the frequency of ppUL83 (pp65) specific PBMCs responding in interferon- γ secretion after peptide stimulation. Data from non-viremic patients are grouped according days post transplantation (< day 50; > day 50). Data from viremic patients grouped according the time relative to the occurrence of HCMV viremia.

Figure 4.7: Scatter-diagram showing the correlation between the pp65 specific interferon- γ and the pp65 tetramer complex frequency in viremic and non viremic patients.

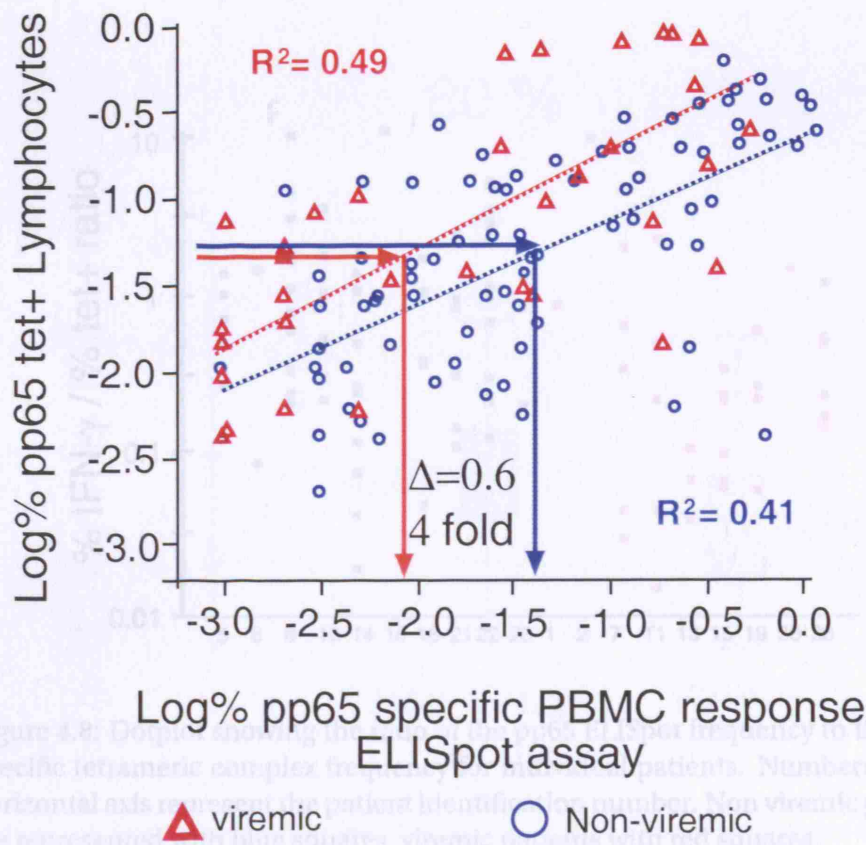


Figure 4.7: Scatter-diagram showing the correlation between the pp65 specific Interferon- γ and the pp65 tetrameric complex frequency in viremic and non viremic patients.

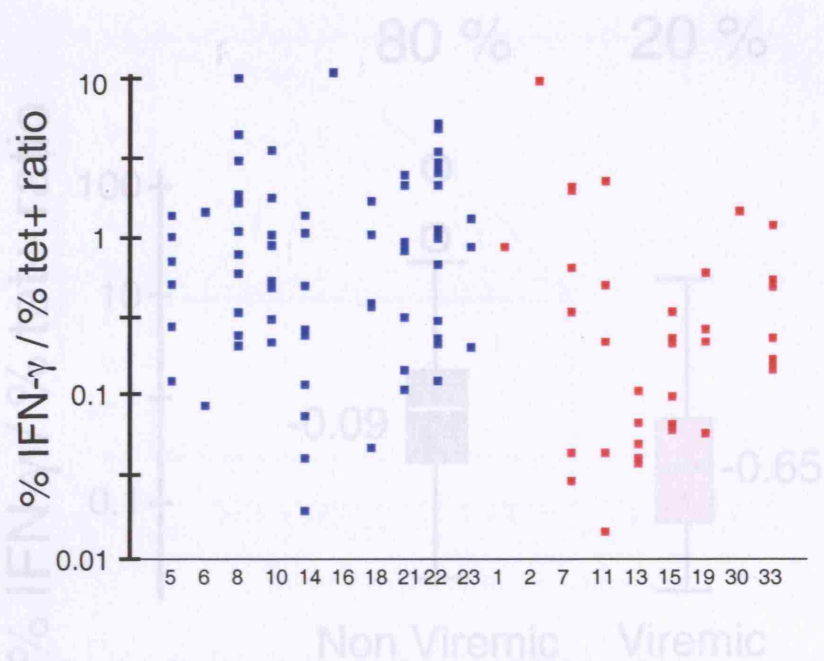


Figure 4.8: Dotplot showing the ratio of the pp65 ELISpot frequency to the pp65 specific tetrameric complex frequency for individual patients. Numbers on the horizontal axis represent the patient identification number. Non viremic patients are represented with blue squares, viremic patients with red squares

specific tetrameric complex frequency in viremic vs. non viremic patients. The % percentages above each plot shows the mean population of tetramer positive cells which are able to secrete interferon- γ .

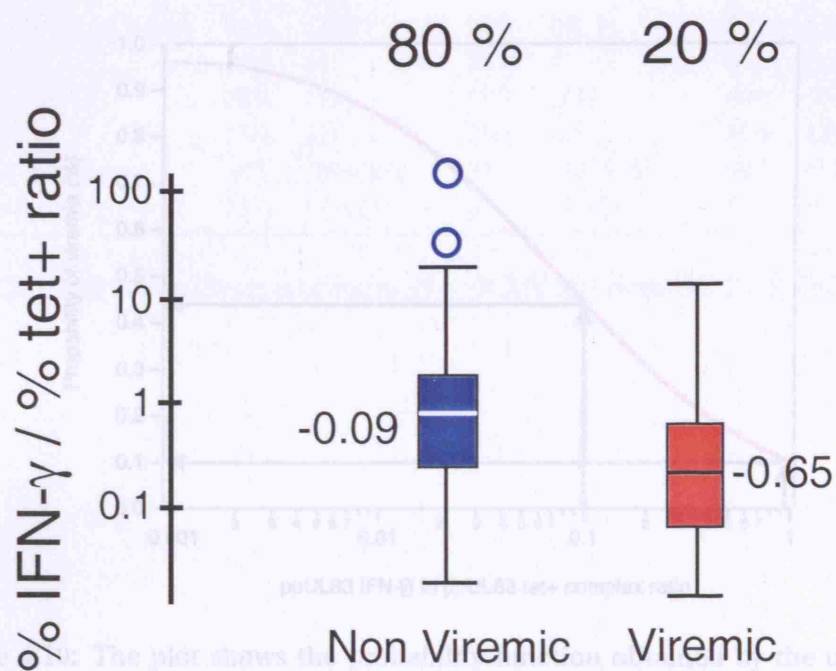


Figure 4.9: The plot shows the univariable linear logistic regression model, predicting viremia based on the IFN- γ to tetrameric complex ratio (see table 4.5 at page 129). The arrows show the increase

Figure 4.9: Boxplots showing the ratio of the pp65 ELISpot frequency to the pp65 specific tetrameric complex frequency in viremic vs. non-viremic patients. The % percentage above each plot shows the mean population of tetramer positive cells which are able to secrete interferon- γ .

Table 4.1: Demographic data of the study cohort

	All patients (N=25)	Non viremic (N=15)	Viremic (N=10)
Male (M/F)	56% (17/8)	57% (10/5)	70% (7/3)
Age (range)	43 (19-67)	29 (19-59)	56 (22-67)
Cardiac (Cardio-lye)	88% (22/25)	93% (14/15)	87% (13/15)
N→N*	14 (56%)	4 (27%)	0 (0%)
N→P or P→P	11 (44%)	11 (73%)	6 (60%)
P→N*	2 (8%)	0 (0%)	2 (20%)
Follow-up (months)	363 (85-684)	263 (68-578)	397 (135-689)
No sampling	13 (7-22)	1 (7-22)	13 (8-16)

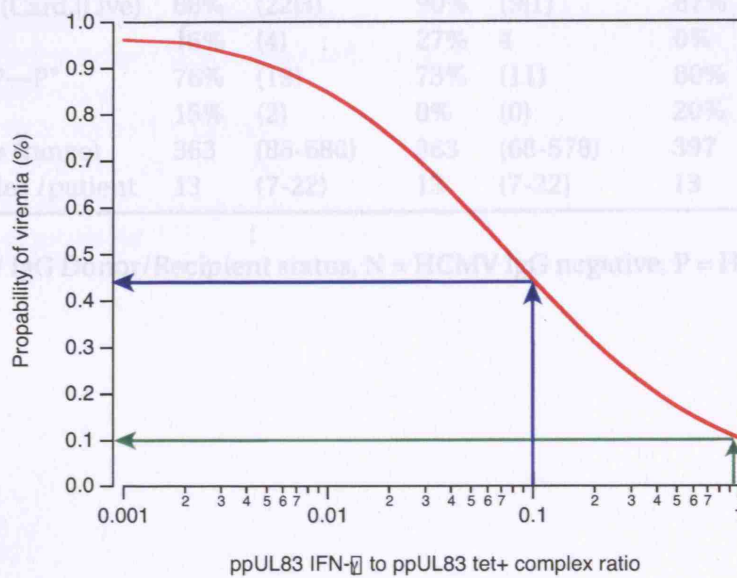


Figure 4.10: The plot shows the probability function obtained by the univariable linear logistic regression model, predicting viremia based on the IFN- γ to tetrameric complex ratio (see table 4.5 at page 129). The arrows show the increase in probability for HCMV viremia from 10% to 42% if the functional capacity is reduced from 100% to 10%.

B7	12%	3	20%	5	16%	4
B8	12%	3	12%	3	0%	0
B9	12%	3	4%	1	4%	2
A7 B8	8%	2	4%	1	4%	1
A2 B8	4%	1	4%	1	0%	0
B7 B8	4%	1	4%	1	0%	0
A2 B7 B8	4%	1	4%	1	0%	0

Table 4.1: Demographic data of the study cohort

	All patients N=25		Non viremic N=15		Viremic N=10	
Male (M F)	68%	(17 8)	67%	(10 5)	70%	(7 3)
Age (range)	43	(19-67)	29	(19-59)	56	(22-67)
Cardiac (Card. Live)	88%	(22 3)	90%	(9 1)	87%	(13 2)
N→N*	16%	(4)	27%	4	0%	(0)
N→P or P→P*	76%	(19)	73%	(11)	80%	(8)
P→N*	15%	(2)	0%	(0)	20%	(2)
Follow up (range)	363	(86-680)	363	(68-578)	397	(135-680)
No samples /patient	13	(7-22)	13	(7-22)	13	(8-16)

* HCMV IgG Donor/Recipient status, N = HCMV IgG negative, P = HCMV IgG positive

Table 4.2: HLA distribution of the study cohort

	All patients N=25		Non viremic N=15		Viremic N=10	
A2	36%	9	20%	5	16%	4
B7	12%	3	12%	3	0%	0
B8	12%	3	4%	1	8%	2
A2 B8	8%	2	4%	1	4%	1
A2 B35	4%	1	4%	1	0%	0
B7 B8	4%	1	4%	1	0%	0
A2 B7 B8	4%	1	4%	1	0%	0

Table 4.3: Immunosuppressive drugs used in the study cohort

Immunosuppressive Drug	Non Viremic (N=15)	Viremic (N=10)
Cyclosporine	4	1
Cyclosporine+Sirolimus	5	1
Cyclosporine+Tracolimus	3	
Cyclosporine +Azathioprine		1
Cyclosporine+Sirolimus+Azathioprine	1	1
Tracolimus		3
Tracolimus+Basiliximab		1
Tracolimus+Sirolimus+MMF	2	
Serolimus+Azathioprine	1	1
not available		1

Table 4.4: Virological markers of the study cohort

	Viremic Patients		Reactivation		Primary HCMV	
	Median	Range	Median	Range	Median	Range
First PCR positive sample	38	15-69	32	15-51	55	42-69
First PCR negative sample	61	30-101	60	30-78	84	67-101
Length of DNAemia	15.5	8-35	15	8-35	20.5	15-26
Treatment length	15.5	8-35	15	8-35	21	15-26
PCR negative after treatment	100%		100%		100%	
PCR negative within 14 days	50%		63%		0%	
Baseline viral load (log10)	3.9	3.1-5	3.9	3-5	4.6	4.3-5
Maximum viral load (log10)	4.1	2.8-6.2	3.9	2.8-6.2	4.6	4.3-5

Table 4.5: Univariable logistic regression model to identify risk factors associated with HCMV viremia

Univariable Models (before viremic or < day 50)			
Risk Factor	Odds Ratio	95% CI	P - value
ppUL83 specific tet+ T-cells (per 10 fold increase)	2.15	1-4.7	0.054
ppUL83 IFN- γ secreting PBMCs (per 10 fold decrease)	3.03	1.6-7.1	0.014
IFN- γ secreting to tet+ ratio (per 10 fold decrease)	6.6	1.5-33	0.01
Age per 1 year	1.07	1.-1.1	0.03

Table 4.6: Frequency of pp65 specific CD8+ T-cells in viraemic and non viraemic patients for different time intervals

Group	Category	No samples	Median (%)	Range
HCMV PCR Negative	< 50 day	55	0.28	0.02-3.35
	> 50 day	50	0.37	0.01-6.92
HCMV PCR Positive	Before	14	0.71	0.29-3.65
	During	10	1.19	0.27-5.2
	Post	45	0.5	0.03-6.46

Table 4.7: Frequency of PBMCs responding after pp65 peptide stimulation with IFN- γ secretion in a ELISpot assay. Frequencies are shown for viraemic and non-viraemic patients

Group	Category	No samples	Median (%)	Range
HCMV PCR Negative	< 50 day	48	0.023	0.005-1.08
	> 50 day	61	0.032	0.005-4.66
HCMV PCR Positive	Before	9	0.027	0.005-0.09
	During	6	0.082	0.005-0.87
	Post	36	0.173	0.005-0.82

4.4 Discussion

This chapter has described the results from 25 patients, of whom 10 were viraemic post renal transplantation. The 10 viraemic and the 15 non viraemic patients were well matched for a series of demographic characteristics, except for age (see table 4.1, page 127). I had hypothesised that patients who developed viraemia would have a Impaired T-cell recognition of HCMV. This hypothesis was clearly rejected because the tetramer assay showed an increased pp65 CD8+ T-cell frequency in patients who were viraemic (see figure 4.5, page 121 and table 4.6, page 130). This unexpected enhanced response was found before individual patients developed HCMV viraemia. This was not a general effect of immunosuppression or receipt of antiviral drugs, since the non-viraemic patients did not show a difference in tetramer frequency when stratified according to less than or more than 50 days post transplantation.

To complement these tetramer studies I used interferon- γ ELISpot assays to investigate the functional potential of these cells. There were no differences in interferon- γ production between the two patient groups (figure 4.6, page 122 and table 4.7, page 130). However, the proportion of HCMV specific T-cells identified by HLA tetramers that could produce interferon- γ was significantly decreased in those patients who were viraemic (figure 4.7, 123). Thus, the second of my two hypotheses i.e. that patients who are viraemic will have evidence of impaired T-cell function was considered proven. The functional defect was seen before the patients became viraemic. The result of the univariable regression model for the ELISpot to tetrameric complex ratio was used to construct a probability plot showing an increase in probability of HCMV viraemia from 10% to 42%, if the functional potential is reduced from 100% to 10% (figure 4.3.7, page 126).

These results are potentially important in two major areas. First, they may shed direct light on why only a subset of patients develops viraemia post transplantation. When the study was designed, I hypothesised that patients would have impaired responses at the time they became viraemic Whereas the results show that the immune deficit precedes the onset of viraemia. This might be explained by low level replication at sites other than the blood (i.e. kidney) priming or expanding CD8+ T-cells, so that they were recognised by tetramers but triggered them inappropriately so that interferon- γ was not produced. An alternative hypothesis would be that a subset of the population has an immune defect specific for HCMV and that this immune defect precedes transplantation. To

test this hypothesis I have recently started to collect samples from haemodialysis patients awaiting renal transplantation. If apparently normal members of the community do have impaired responses to HCMV this would be an unexpected observation. One possible explanation might be that children who acquire congenital or perinatal infection may have a persisting immune defect which is still present at the time they require transplantation many years later. Since approximately 5% to 20% of patients acquire perinatal infection in many communities in the world, it is tempting to speculate that this might be a possible explanation for these findings. However, prospective studies would be required to test this and these would require many years of follow-up from perinatal infection to the time of requiring transplantation.

Second, it is possible these results may shed some light on the immune senescence, which has been described in elderly patients [181]. I observed that patients who developed HCMV viraemia were older (figure 4.1, page 117). It has been suggested that low level HCMV replication over time leads to the accumulation of large numbers of HCMV specific CD8+ T-cells with an inappropriate response to the presence of HCMV [194, 193]. Some authors have described oligoclonal bands in elderly patients attributed to HCMV specific T-cells, and it would be interesting to examine whether the phenotype and functional type of these cells matches that described in this chapter.

Chapter 5

A randomized, controlled trial comparing ganciclovir or ganciclovir plus foscarnet (each at half dose) for pre-emptive therapy of cytomegalovirus infection in transplant recipients

5.1 Introduction

Human Cytomegalovirus (HCMV) is a common infectious agent, which rarely causes symptoms unless the patient is immunocompromised. Following transplantation of bone marrow or solid organs, HCMV can cause fever, pneumonitis, hepatitis, enteritis, or retinitis, collectively termed 'HCMV disease'. Natural history studies show that HCMV viremia precedes HCMV disease and that the peak viral load correlates strongly with the development of HCMV disease [40, 41]. In multivariable statistical models, peak viral load explains the previously identified risk factors of donor/recipient serostatus [40, 91]. Management strategies for preventing HCMV disease include giving antiviral prophylaxis to patients from the time of transplant onwards (reviewed in [110]), or using the results of virologic surveillance to identify asymptomatic patients with viremia and offering them antiviral treatment (reviewed in [60]) before disease develops (pre-emptive therapy). In this institution, bone marrow, liver and renal transplant patients are tested twice weekly by PCR. Pre-emptive therapy with ganciclovir (5mg/kg bd) is given intravenously for 14 days [65] to patients with two consecutive PCR positive results. Ganciclovir-induced neutropenia is managed by switching to foscarnet, a drug which is nephrotoxic and causes electrolyte imbalances [128]. Ganciclovir and foscarnet show in vitro synergistic activity against HCMV [172], and a randomised trial in AIDS patients showed significantly delayed progression of HCMV retinitis in patients who received this combination [261]. We therefore hypothesised that a combination strategy consisting of half-dose ganciclovir and half-dose foscarnet may provide more efficacious control of HCMV viremia in the transplant setting while reducing the risk of severe side-effects when used for pre-emptive therapy. This chapter reports the results of a randomized, controlled trial designed to test this hypothesis and to investigate the virologic determinants of treatment outcome.

5.2 Material and Methods

The general management of patients undergoing bone marrow, liver or renal transplantation at our center is described in detail elsewhere [40, 41, 91]. Of note, no HCMV anti-viral prophylaxis is given to recipients of solid organs, whereas all bone marrow patients receive high-dose aciclovir according to a previously published protocol [217].

5.2.1 HCMV surveillance

All patients had whole blood samples collected twice weekly and tested for CMV DNA by a PCR method previously described [147]. The sensitivity of the assay is 200 genomes/ml (gen/ml) of blood, a value which has been shown to identify patients at risk of future HCMV disease [147]. All HCMV positive samples were quantified using a quantitative-competitive PCR described in detail in 2.9.2, page 82. These quantitative values were not available to clinicians treating the patients enrolled in the study.

5.2.2 Randomised trial design

Patients with two consecutive HCMV blood PCR-positive samples for the first time after transplantation were invited to enter the randomized trial, which was approved by the local Ethics Committee (Institutional Review Board). Patients were randomized to receive either full-dose ganciclovir (5 mg/kg bid) or half-dose ganciclovir (5 mg/kg od) plus half-dose foscarnet (90 mg/kg od) for 14 days. Doses were adjusted according to renal function. Patients received granulocyte colony stimulating factor (G-CSF) at the discretion of the physician. Randomization was stratified by type of organ transplanted. The trial was administered using sealed envelopes containing the randomization code that were opened once the patient gave informed consent. Patients with impaired renal function (creatinine clearance <30 ml/minute), neutropenia ($< 0.5 \times 10^9 / L$) or HIV infection were excluded from the study. Patients on either arm of the study who failed to clear their HCMV viremia within 14 days of treatment could either be withdrawn from the study or be given a further course of treatment at the discretion of the physician. The first patient was enrolled from December 1998, and the last in February 2001. Patients who developed serious adverse events related to toxicity profiles of ganciclovir (neutropenia) or foscarnet (electrolyte disturbance, renal impair-

ment) could be withdrawn from the study, switched to the other arm or continue on the study drug with a reduced dose.

5.2.3 Statistical analysis

The primary end-point was the proportion of patients who became PCR-negative within 14 days of starting study medication. Secondary end-points addressed the safety and tolerability of combination therapy and changes in HCMV load during therapy. All analyses were by intention to treat. Comparison of continuous variables such as viral load between groups was achieved using the two-sided t-test. Mann Whitney U test was used for the comparison of viral replication rates. The rate constant κ for viral growth or decay before or after therapy was computed assuming exponential growth/decay as described elsewhere [62]. Doubling times and half life of decline were calculated using the following formulae $T_d = \ln(2)/\kappa$ and $T_{\frac{1}{2}} = \ln(2)/\kappa$. Simple linear least squares fit regression modes was used for analysing the relationship between viral replication kinetics and viral load. Factors associated with the primary endpoint were modeled with a univariable and multivariable logistic regression model using intention to treat design. Kaplan-Meier plot and a log-rank test were used to test the hypothesis that both treatment arms were equal.

Important practical difficulties in the co-administration of these two compounds were anticipated. Specifically, foscarnet and ganciclovir are incompatible in the same dilution fluid, and the low solubility of foscarnet increased the fluid challenge the patients were subjected to. For these reasons, it was decided that only a substantial superiority of the combination would be clinically significant. Based upon historical data, 50% of patients given ganciclovir monotherapy were expected to become PCR negative after 14 days of therapy. A study size of 48 patients (24 in each group) had the power to detect a statistically significant ($p < 0.05$) increase in this rate to 90%. Data were analysed with the computer program R [125].

5.3 Results

5.3.1 Baseline characteristics

Details of the patients randomized to each treatment arm are given in Table 5.1. The patients were well matched for sex, age and HCMV donor/recipient serostatus. Viral loads at day 0 (baseline viral load) were similar in both treatment arms (GCV: 4.28 \log_{10} ge/ml vs GCV + FOS: 4.01 \log_{10} ge/ml; Mann-Whitney U test

p=0.73). There was no difference between the time of the second HCMV PCR positive sample and initiation of therapy when patients were stratified according to treatment allocation (median 4 days for GCV / 5 days for GCV+FOS, Mann-Whitney U test p= 0.24), or transplant type (BMT: median 4 days; solid organ: 5 days, Mann-Whitney U test p=0.19)

5.3.2 Assessments of virological responses

The primary end-point of PCR negativity (<200 ge/mL) was reached by 71% (17/24) of patients randomized to ganciclovir and 50% (12/24) of patients randomized to combination therapy (Chi-square test p= 0.14). Among these who reached the primary study endpoint, the median time to become blood HCMV PCR negative was 6 days in the GCV arm compared to 5.5 days in the combination arm (median times of 6 days vs 11 days when considering all patients using a Kaplan-Meier approach; Table 5.2). A Kaplan-Meier plot illustrating the proportion and times when patients became HCMV PCR negative within the first 14 days of therapy is shown in figure 5.1. Three patients in the GCV treatment arm and one patient in the combination arm had already become HCMV PCR negative at day 0. Two patients (both in the combination arm) had no negative HCMV PCR results within 50 days. Antiviral therapy was stopped in one patient (GCV arm) at day 12. Statistically, there was no difference between the two treatment arms for the primary endpoint. (log-rank test, p=0.19). With respect to drug toxicity, 7 patients experienced toxicity, all of whom were in the combination arm (7/24 in the combination arm versus 0/24 in the GCV arm, Fisher's exact test p=0.009; table 5.2).

Table 5.1: Characteristics of patients according to treatment assignment

Characteristics	Treatment allocation	
	GCV	GCV+FOS
Patients randomized	24	24
Bone marrow	8	9
Liver	8	9
Renal	8	6
Age (years)		
Median	50	45
Range	2-68	2-56
Sex (Male:Female)	15:09	14:10
HCMV IgG Serostatus ¹ Donor Recipient ²		
D+R-	2	(1,1,0) 3 (0,3,0)
D-R+	4	(1,2,1) 3 (0,3,0)
D+R+	15	(5,4,5) 15 (7,5,3)
D-R-	1	(0,1,0) 0 (0,0,0)
Baseline Viral Load (log gen/ml)		
Median	4.3	4
Range	2.7-5.9	3.0-6.2

¹ Donor HCMV IgG serostatus unknown in two patients (both renal)

² in bracket number of patients according the transplant group; BMT, Liver and Renal

Table 5.2: Number of patients achieving primary and secondary end-points of the study according to treatment assignment.

Characteristics	Treatment allocation		Significance
	GCV N (%)	GCV+FOS N (%)	
End-point			
Primary			
HCMV PCR negative within 14 days therapy	17 (71%)	12 (50%)	0.12 ¹
Secondary			
Patients stopping or reducing dose due to toxicity in 14 days of randomized therapy	0 (0%)	7 (29%)	0.009 ²
Median time to HCMV PCR negative (days)			
Among those reaching primary end-point	6	5.5	
Among all patients	6	11	
Patients developing a second episode of HCMV viremia after successful treatment ³			
Among those reaching primary end-point	1	4	0.35 ²
Among all patients	6	9	0.53 ²

¹Chi-square test

²Fisher's exact test

³within 365 days after treatment

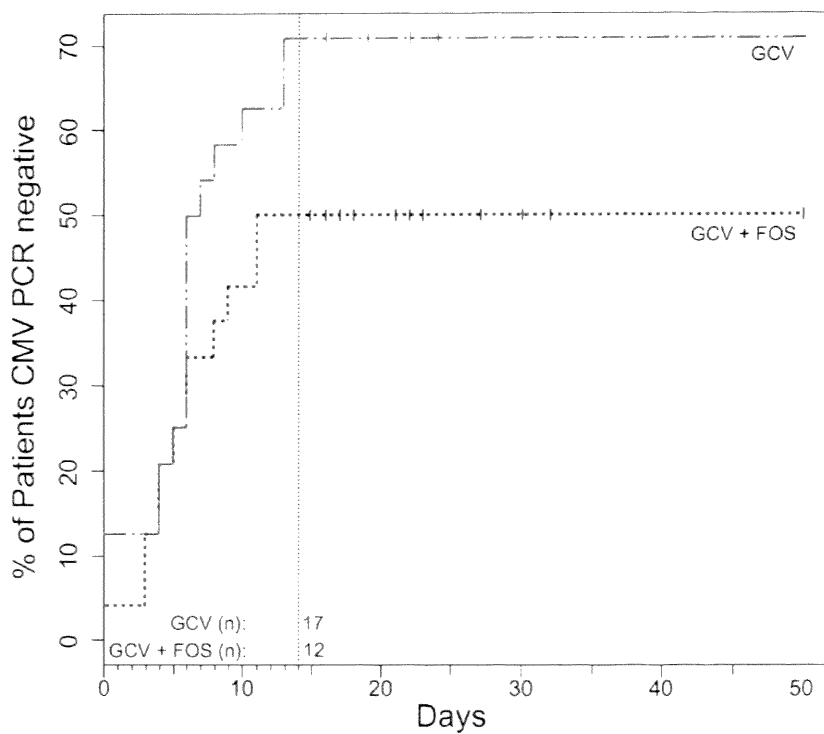


Figure 5.1: Kaplan-Meier analysis of the time to reach the primary endpoint for patients randomized to the GCV or GCV+FOS treatment arm. The vertical dashed line at day 14 indicates the primary endpoint of the study. Crosses after 14 days indicate when a patient who failed the study endpoint became HCMV negative.

5.3.3 Assessment of drug toxicity during study period

All cases of switching and dose reduction due to toxicity occurred in the solid organ transplant group (4 liver and 2 renal transplant recipients), while one patient in the BMT group was withdrawn from the study (GCV+FOS arm) at day 12 because of failure to engraft their donated marrow. In six patients, who continued on the study, toxicity is compatible with the toxicity profile of foscarnet. Three out of these patients were swapped to ganciclovir, and in three patients the dose was reduced (Table 5.3).

Table 5.3: Number of patients with reported drug toxicities during the study period, clinical management and study outcome.

Transplant Type	Randomized Drug	Drug of toxicity	Abnormal clinical parameter	Management ¹	Study outcome
BMT	GCV+FOS	12	Failure to engraft	Withdrawn	Failure
Liver	GCV+FOS	4	Mg ²⁺ ↓	→ GCV	Failure
Liver	GCV+FOS	4	Creatinine ↑	→ GCV	Failure
Liver	GCV+FOS	6	Creatinine ↑	→ GCV	Failure
Liver	GCV+FOS	9	Creatinine ↑	Dose ↓	No failure
Renal	GCV+FOS	11	Creatinine ↑	Dose ↓	No failure
Renal	GCV+FOS	11	Creatinine ↑	Dose ↓	No failure

¹ → change of study drug to ↓ dose reduction of study drug

5.3.4 Virologic predictors of the outcome of therapy

In 42 of the 48 patients, I was able to measure virologic parameters, such as virus load at initiation of therapy, viral growth before therapy, and viral decay rates during therapy. Patients who failed to reach the primary end point had a higher virus load at baseline (difference, 0.77 log₁₀ genomes/mL; P = 0.02, t-test; 5.2). Although there was no difference in the viral replication rate (for ganciclovir, 0.33 day⁻¹ vs. for ganciclovir plus foscarnet, 0.43 day⁻¹; P = 0.91, Mann-Whitney U test) or viral decay rate (for ganciclovir, -0.42 day⁻¹ vs for ganciclovir plus foscarnet, -0.28 day⁻¹; P = 0.26, Mann-Whitney U test) in virus load, between the two therapy arms, the viral decay rate was significantly higher in patients who reached the primary end point than in those who failed to control replication to <200 genomes/mL at day 14 (for no failure, -0.61 day⁻¹ vs. for failure, -0.24 day⁻¹, corresponding to half-life of decay of 1.1 days and 2.9 days, respectively; P < .0002; figure 5.3). Similar results in viral decay rates were obtained when patients were analyzed according to transplant type (bone marrow: no failure, -0.64 day⁻¹ vs. for failure, -0.26 day⁻¹; solid organ: no failure, -0.69 day⁻¹ vs. for failure, -0.23 day⁻¹). Analyses of the viral replication kinetics before therapy revealed that patients who failed to reach the primary end point had a significantly faster HCMV growth rate (for failure, 0.46 day⁻¹ vs. for no failure, 0.26 day⁻¹; P < 0.002; figure 5.4). These growth rates correspond to viral doubling times of 1.51 days and 2.7 days, respectively.

The correlation between viral decay rates and baseline virus load, for patients who reached or failed to reach the primary end point, are shown in figure 5.5. There was a significant correlation between baseline HCMV load and decay rate in each group (r² for failure, 0.6 vs. r² for no failure, 0.52; P < 0.001). In addition, when patients were stratified according to whether they had reached the primary end point, there was a strong correlation between the viral growth rate and the viral decay rate after therapy (r² for failure, 0.54 vs. r² for no failure, 0.73; P = 0.001; see figure 5.5, page 148).

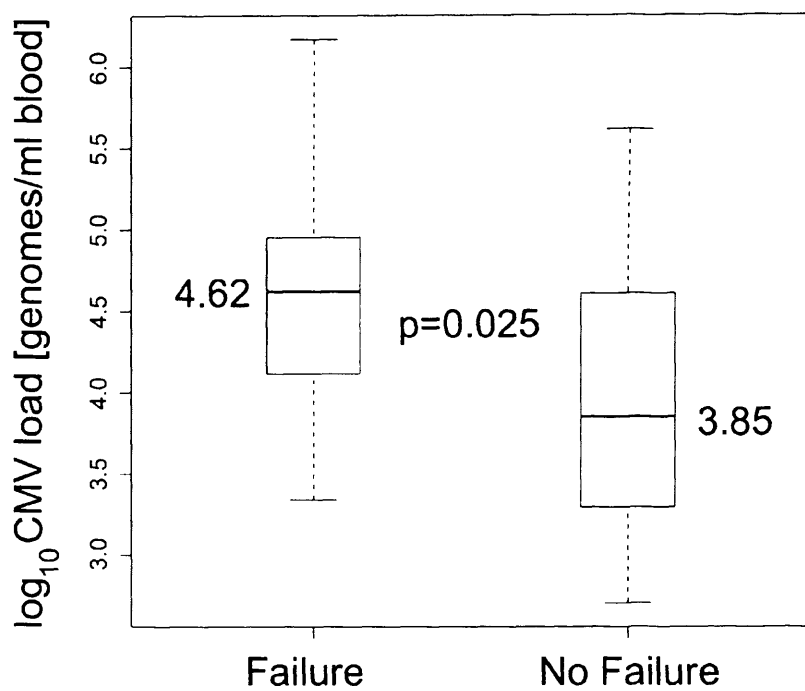


Figure 5.2: Box-plots illustrating the relationship between baseline viral loads for patients who became HCMV negative within 14 days (no failure) and patients who failed the primary study endpoint. Horizontal lines display the 10th, 25th, 50th (thick line), 75th and 90th percentiles; with box encompassing 50% of values.

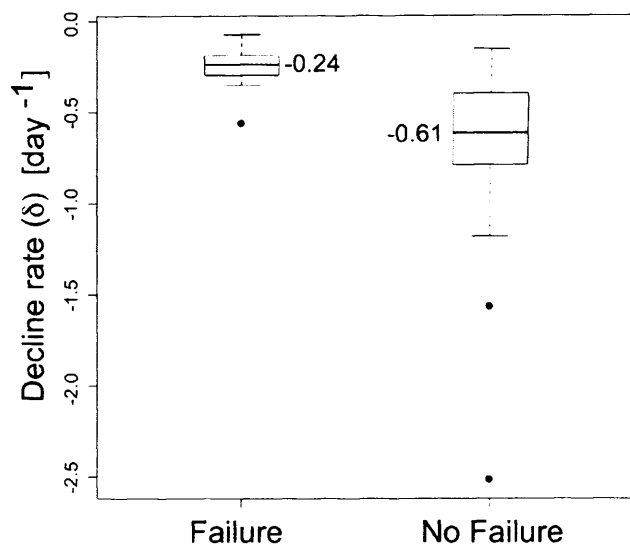


Figure 5.3: Box-plots showing the decline rate (κ), after initiating antiviral treatment in patients who failed or did not fail the primary endpoint. Decline rate were statistically each significantly different in patients who failed the primary endpoint compared with patients who did not fail the endpoint of the study. Horizontal lines display the 10th, 25th, 50th (thick line), 75th and 90th percentiles; with box encompassing 50% of values.

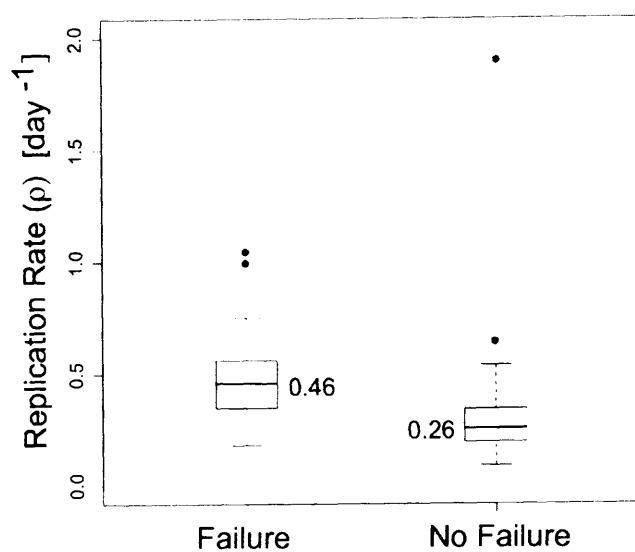


Figure 5.4: Box-plots showing the replication rate (ρ), before initiating antiviral treatment in patients who failed or did not fail the primary endpoint. Replication rates were statistically each significantly different in patients who failed the primary endpoint compared with patients who did not fail the endpoint of the study. Horizontal lines display the 10th, 25th, 50th (thick line), 75th and 90th percentiles; with box encompassing 50 of values.

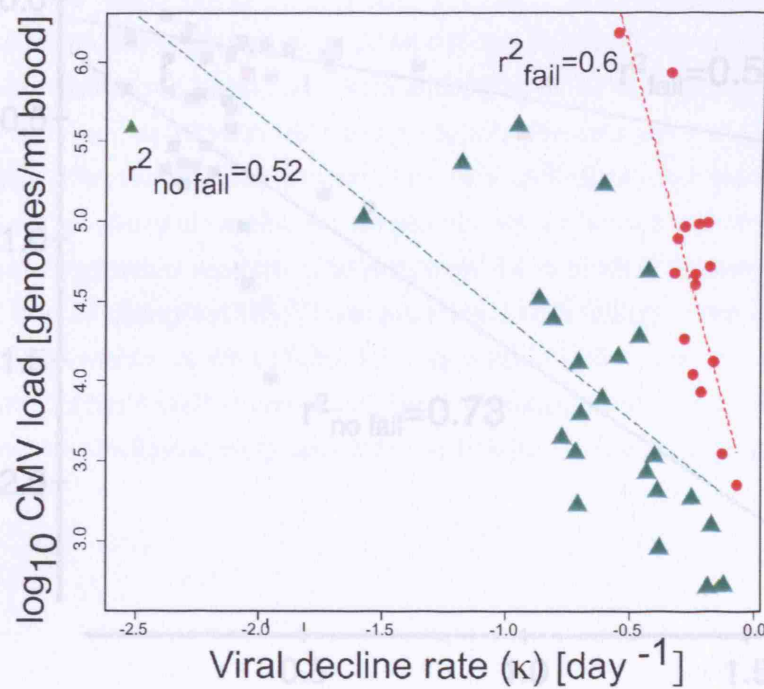


Figure 5.5: Scatterdiagram showing the correlation between viral decline rate (κ) and HCMV baseline viral load. Data are stratified according the study endpoint. A correlation line was fitted for each strata and the goodness of fit expressed as an r^2 value.

5.3.5 Quantifying the risk associated with poor antiviral response

Univariable linear regression models were used to identify factors associated with the primary endpoint (HCMV PCR negative within 14 days). In these models, higher viral load on the day of initiating antiviral therapy (odds ratio (OR) 2.39, $CI_{95\%}$: 1.05-5.45), faster viral doubling time (t_D ; OR: 2.05, $CI_{95\%}$: 1.28-6.82) prior to therapy and slower half-life of decline in viral load after therapy ($t_{1/2}$; OR 3.01, $CI_{95\%}$: 1.45-6.25) were all associated with failure to reach the primary end-point.

Age (<40 years), gender, transplant type (BMT vs solid organ), study drug (vs combination) and time to therapy delay did not appear to be associated with the primary endpoint (Table 5.4). As a consequence of the correlation between viral load at initiation of therapy, replication rate prior to therapy and decline rate after therapy, multivariable models including all these factors did not provide meaningful results. Consequently, we undertook a series of bivariable logistic regression models. The results of these models illustrates, that in all cases, type of transplant (BMT) was associated with failing to become HCMV PCR negative within 14 days (Table 5.5). In addition, the measure of the viral replication (higher baseline viral load, faster doubling time or slower viral half-life) was independently associated with failure to reach the primary study

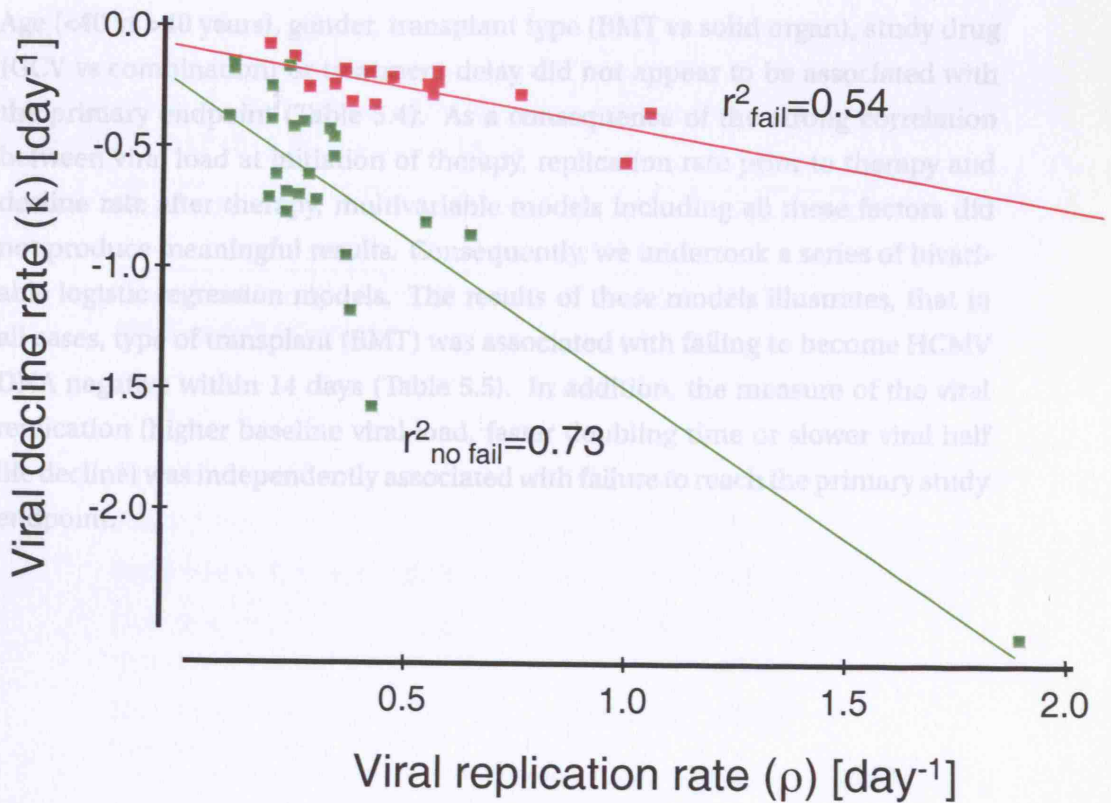


Figure 5.6: Scatterdiagram showing the correlation between viral replication rate (ρ) and viral decline rate (κ). Data are stratified according the study endpoint. A correlation line was fitted for each strata and the goodness of fit expressed as an r^2 value.

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Table 5.4: Univariable analysis of risk factors associated with primary endpoint becoming HCMV DNA PCR neagative by 14

Risk factor	Odds ratio	CI _{95%} for odds ratio	Significance
Transplant Group (BMT vs Solid Organ)	2.36	0.70–7.94	0.17
Viral load ¹ (per log ₁₀ higher)	2.39	1.05–5.44	0.038
Age (<40 vs >40)	1.19	0.37–3.86	0.77
Study drug	0.41	0.13–1.35	0.144
Sex (male vs female)	0.59	0.18–1.91	0.37
Doubling time (per day decrease)	2.95	1.28–6.82	0.01
Half life of decline (per day increase)	3.01	1.45–6.25	0.003

¹Viral load measured at the day of initiation of therapy

Table 5.5: Multivariable analysis of risk factors associated with primary endpoint becoming HCMV DNA PCR neagative by day 14

Model	Risk factor	Odds ratio	CI _{95%} for odds ratio	Significance
1	Transplant Group (BMT vs Solid Organ)	3.94	0.92–16.92	0.064
	Viral load ¹ (per log ₁₀ higher)	2.96	1.01–6.15	0.048
2	Transplant Group (BMT vs Solid Organ)	3.79	0.78–18.24	0.09
	Doubling time (per day decrease)	3.02	1.28–7.14	0.018
3	Transplant Group (BMT vs Solid organ)	7.46	1.19–46.71	0.031
	Half life of decline (per day increase)	3.7	1.55–8.84	0.003

¹Viral load measured at the day of initiation of therapy

5.3.6 Subsequent episodes of HCMV viremia

We observed in 31% (15/48) of the patients a second episode HCMV viremia within one year after finishing the first course of treatment. There was no significant difference in the number of patients who experienced a second episode of HCMV viremia, when analysed according the initial randomized drug (GCV=6, GCV+FOS=9; Fisher's exact test $p=0.5$; table 5.6). However, patients who failed the primary endpoint of the study were more likely to have a second episode of HCMV reactivation (table 5.7 Fisher's exact test $p=0.01$). In addition there was a trend for higher baseline viral load and faster replication rate prior to therapy among patients with a second episode of viremia.

Table 5.6: Frequency of a second episode of HCMV reactivation according treatment allocation.

N=48	Second HCMV reactivation	
	Yes	No
GCV	6	18
GCV plus Fos	9	15

Fisher' s exact test p=0.5

Table 5.7: Frequency of a second episode of HCMV reactivation stratified according HCMV negative at 14 days.

N=48	Second HCMV reactivation	
	Yes	No
HCMV negative within 14 days	5	24
Not HCMV negative within 14 days	10	9

Fisher' s exact test p=0.01

5.4 Discussion

The main conclusion of this randomized, controlled clinical trial was that the combination of half dose GCV and FOS therapy was not superior to ganciclovir monotherapy for pre-emptive therapy in transplant recipients. The decline rate of virus load after therapy was very similar between patients randomised to receive GCV or the combination therapy. However, there was a trend in favour of the ganciclovir arm with respect to the primary end-point of PCR negativity by day 14. Thus, the lack of a significant benefit to the combination of GCV and FOS could not be explained by inadequate study power. Since patients were well matched at baseline for all known prognostic variables, including donor/recipient serostatus and baseline viral load, this outcome was surprising. The hypothesis that the combination might be superior to ganciclovir monotherapy was based upon two previously published observations; ganciclovir plus foscarnet are synergistic in vitro [172] and the combination proved superior in controlling HCMV retinitis in AIDS patients [261]. However, we now know that extrapolation of data from in vitro studies with anti-HCMV compounds can be misleading since aciclovir, which has a poor efficacy in vitro, has been shown to be efficacious in vivo within a series of randomized, controlled clinical trials [11, 167]. Furthermore the results of the randomized, controlled trial of GCV and FOS in AIDS retinitis patients, which showed a clear superiority of the combination arm, were obtained in a patient group who had experienced high level HCMV replication and had been pre-treated with GCV. We have previously shown that the rapid dynamics of HCMV coupled with prolonged persistence of low levels of GCV can result in a rapid flux of wild type and drug resistant viral populations and also that in vitro culture of viruses in the absence of GCV can result in an under-appreciation of the quantity of resistant viruses present in the clinical inoculum [62, 64]. Thus, a high incidence of unrecognised resistance to ganciclovir could explain the observed differences between the trials, with the GCV + FOS arm showing improved virologic and clinical benefit in AIDS retinitis patients due to the ability to control low level GCV resistant strains of viruses with mutations in the UL97 gene. In the current study, the availability of viral load data enabled us to investigate virological parameters associated with treatment failure. Patients who failed to control replication to low levels (<200 ge/ml) within 14 days of therapy had a significantly higher baseline load, a much faster growth rate of virus prior to therapy (average $t_{\frac{1}{2}} = 1.5$ days) and also a much slower rate of decline following therapy

(average $t_{\frac{1}{2}} = 2.9$ days) compared to patients who reached the primary endpoint. These observations on the importance of rate of viral load increase, and decline following therapy, in patients with poor response to antiviral treatment have important practical implications and further studies are required to determine the role of host parameters in facilitating the removal of virally infected cells after starting therapy. It is known that a subset of patients managed via pre-emptive therapy will experience a recurrence of their viremia. In the present study this constituted 31% of the cohort. Patients who failed to reach the primary endpoint of the study were more likely to have a second episode of HCMV DNAemia within the first year (Fisher's exact test $p=0.013$). In addition, patients who had a second reactivation episode had a higher viral load at baseline, a higher replication rate prior to antiviral treatment and a slower decline rate after starting antiviral treatment. This latter observation is similar to those of Humar and colleagues [124] who showed that solid organ transplant recipients who had second episodes of viremia had a $t_{\frac{1}{2}}$ of 8.8 days after iv GCV therapy compared to 3.17 days for patients with a single HCMV viremic episode. In conclusion, this trial shows that combination antiviral therapy for HCMV viremia with GCV + FOS does not appear to control viral replication better than GCV monotherapy. All observed drug toxicity occurred in patients randomized to the combination therapy. Nevertheless, combination therapy is one management option colleagues should consider when faced with a patient unable to tolerate full doses of ganciclovir; ie combination therapy may help manage the neutropenia associated with the full dose GCV, but does not offer superior potency.

Chapter 6

Kinetics of cytomegaloviral load decline in solid organ transplant recipients after pre-emptive valganciclovir therapy

6.1 Introduction

Human cytomegalovirus (HCMV) remains an important cause of morbidity following solid organ transplantation. Infection has been associated with a number of direct and indirect effects in the immunocompromised host including hepatitis, prolonged pyrexia, acute and chronic graft rejection [164]. HCMV replicates rapidly in the human host and viral load is directly related to the probability of disease development [40, 41, 62]. GCV therapy has become one of the most commonly used antivirals to control HCMV replication in solid organ transplant recipients. Therapeutic approaches include prophylaxis, usually targeted to patients at high risk of developing HCMV disease, and pre-emptive therapy where drug is administered on the basis of detection of active HCMV replication using sensitive laboratory methods such as the antigenemia assay or PCR. Recently, controlled clinical trials of the valine ester prodrug of GCV, VGCV, have shown that it is effective at controlling HCMV replication and disease in HIV-infected patients and in high risk solid organ transplant patients [175, 18, 201]. VGCV offers many advantages over existing formulations of GCV since high plasma levels of drug can be achieved through oral dosing and patients no longer require extensive hospitalisation for intravenous infusions. A dose of 900mg of VGCV provides similar plasma GCV exposure as a 5 mg/kg dose of iv GCV [206, 205]. At present there have been no trials comparing pre-emptive therapy with VGCV or iv GCV in solid organ transplant recipients. However, increasingly VGCV is being used in such a treatment modality. We therefore investigated whether the kinetics of control of HCMV replication in patients receiving VGCV were comparable to those we have previously observed in patients receiving iv GCV [177].

6.2 Methods

6.2.1 Patient selection

In a retrospective study, renal or liver transplant patients receiving either oral VGCV (900 mg bd) or iv GCV (5 mg/kg bd) for the treatment of HCMV infection were identified between October 2001 and September 2003. The dose of VGCV and GCV was adjusted according to the renal function of the patient as recommended by the manufacturer.

6.2.2 HCMV monitoring and HCMV quantification

Patients were monitored for HCMV DNAemia at least twice weekly when hospitalized, or whenever attending the outpatient clinic. HCMV infection was defined as two consecutive positive HCMV PCR results (cut-off 200 genomes/ml). HCMV PCR was carried out on a routine basis with an in-house TaqMan (ABI) based method adapted from our previously published method [77], as described in 2.9.3, page 82.

6.2.3 Antiviral therapy

Antiviral HCMV therapy was recommended after two consecutive positive HCMV PCR samples and was continued until two consecutive negative HCMV PCR samples were obtained. The choice of anti HCMV drug was made by the treating physician.

6.2.4 Calculation of viral kinetics and statistical analysis

The length of a viremic episode was defined as the time interval between the last negative HCMV PCR sample, followed by at least two consecutive positive results until the first negative PCR sample. Treatment delay was defined as the time from the first positive PCR sample until initiating antiviral therapy. Baseline viral load was defined as the viral load measured at time of starting antiviral therapy. A linear curve fit through all available viral load data prior to antiviral therapy and after antiviral therapy was performed. Doubling time or half life were calculated using standard exponential growth or decay functions. Groups with continuous variables were compared with Mann-Whitney U test. Difference in proportions between groups were calculated with Fisher's exact test. All statistical analysis was performed as intention to treat analysis with the software R [125].

6.3 Results

During the period from September 2001 to October 2003, 22 patients (15 liver and 7 renal transplant recipients) received pre-emptive therapy with VGCV (900 mg bid). During the same time period a further 23 patients (11 renal and 12 liver transplant) received pre-emptive therapy with iv GCV (5mg/kg bid), table 6.1 page 162. In all patients studied, HCMV loads were determined twice a week

prior to and during therapy. With the exception of two patients (both iv GCV), patients received antiviral therapy until becoming HCMV PCR negative. A total of 107 samples were quantified before initiating antiviral therapy (median of 3 per patient) and a further 177 HCMV PCR positive samples were quantified after starting antiviral therapy (median of 4 per patient).

The demographic characteristics of the two treatment groups are well matched in terms of allocated patients (23 GCV vs 22 VGCV), age (mean 43.8 years GCV vs 49.3 years VGCV) and gender (GCV: 14 male/ 9 female, VGCV: 9 male/13 female). More primary HCMV infections (donor HCMV positive (D+), recipient HCMV negative (R-)) received GCV (n=7) compared to VGCV (n=2), however this difference did not reach statistical significance (Fisher's exact test $p=0.27$); table 6.1. Treatment groups were well matched for D-R+ (4 GCV, 4 VGCV) and D+R+ (12 GCV, 14 VGCV) combinations. Virological parameters before starting antiviral therapy, were comparable for viral load at treatment initiation ($3.55 \log_{10}$ genomes/ml GCV vs $3.81 \log_{10}$ genomes/ml VGCV, $p=0.67$), peak virus load ($3.87 \log_{10}$ genomes/ml GCV vs $4.15 \log_{10}$ genomes/ml, $p=0.68$) and doubling time (2.03 days GCV vs 1.82 days VGCV, $p=0.47$). The treatment delay was 8 days in the GCV group and 9 days in the VGCV group ($p=0.98$); see table 6.2, page 163.

Following therapy, patients receiving VGCV had a median half life of decline of 2.16 days compared with a half life of decline in patients receiving iv GCV of 1.73 days ($p=0.63$). The median decline in HCMV load at day 7 was $-1.07 \log_{10}$ genomes/ml in patients receiving VGCV compared to $-0.65 \log_{10}$ genomes/ml in patients receiving iv GCV, although this difference did not reach statistical significance ($p=0.14$). At day 14, viral load decline was similar between both treatment groups (GCV: $-1.17 \log_{10}$ genomes/ml vs VGCV: $-0.98 \log_{10}$ genomes/ml, $p=0.92$). Overall, time to become HCMV PCR negative was comparable between the two groups (median 14 days for GCV vs 15.5 days for VGCV, $p=0.86$). The data are summarized in table 6.3 on page 164.

Individual decline profiles for patients receiving iv GCV or VGCV pre-emptive therapy are shown in figure 6.1, page 165. A composite figure summarizing all patients is shown in figure 6.2, page 166. As noted in the table 6.3 the decline rates of HCMV load between the two treatment groups were almost identical.

6.4 Discussion

In the absence of data from controlled clinical trials of VGCV, I have used a single centre experience of pre-emptive therapy to compare the post therapy kinetics of HCMV replication in solid organ transplant recipients receiving either VGCV or iv GCV. Since VGCV use was becoming more widespread in the pre-emptive therapeutic setting it was important to show that the ability to control HCMV replication rapidly and effectively with valganciclovir was comparable to that achieved with iv GCV. The results clearly show that VGCV (900 mg bid) and iv GCV (5mg/kg bid) produce similar efficacy levels for the control of HCMV replication in this therapeutic setting. These results concur with the pharmacokinetics of VGCV observed in liver transplant and HIV-infected patients i.e. a 900mg dose of VGCV produces comparable plasma GCV levels to a 5mg/kg iv dose of GCV. We have previously shown that replication events occurring prior to the initiation of pre-emptive therapy can have a substantial effect on the observed response to therapy [139]. Hence, rapid viral replication (a fast doubling time) and high viral load at the start of therapy are both associated with a slower rate of decline following therapy. Such events are consistent with the non-steady state viral dynamics which are apparent in the time period where viral loads traverse 200 genomes/ml to 10,000 genomes/ml. In the analyses presented here, viral load at the initiation of therapy and the average doubling time of virus prior to therapy were well matched between patients who received VGCV or iv GCV. This study therefore provides the first detailed assessment of HCMV kinetics following pre-emptive VGCV therapy in solid organ transplant recipients and emphasises the rapid control of HCMV replication achievable. The inclusion of measures of viral dynamics into future clinical trials of VGCV in other clinical settings will facilitate our understanding of the factors required for the successful control of HCMV in transplant recipients.

Table 6.1: Patients characteristics according to treatment

Characteristics	Treatment allocation	
	iv Ganciclovir	Valganciclovir
Patients allocated	Total 23	22
	Liver 12	15
	Renal 11	7
Age (<i>years</i>)	Mean 43.8	49.3
	Range 28–65	35–66
Sex (Male:Female)	14:9	9:13
CMV IgG Serostatus Donor Recipient		
	D+R- 7	2
	D-R+ 4	4
	D+R+ 12	14
	D-R- 0	0
	ND 0	2

Table 6.2: Virological parameter before starting antiviral therapy

Characteristics	Treatment allocation		Significance
	iv GCV	VGCV	
Baseline Viral Load ($\log_{10} \text{gen} \times \text{ml}^{-1}$)			
n=	22	22	
mean	3.77	3.85	p=0.78 ¹
median	3.85	3.81	
range	2.29-5.75	2.37-5.52	
Peak Viral Load ($\log_{10} \text{gen} \times \text{ml}^{-1}$)			
n=	23	22	
mean	4.13	4.18	p=0.85 ¹
median	3.87	4.15	
range	2.92-5.76	3.01-5.52	
Replication rate $\text{gen} \times \text{ml}^{-1} \times \text{day}^{-1}$			
n=	21	21	
mean	0.40	0.49	p=0.44 ²
median	0.34	0.38	
range	0.11-1.06	0.12-2.2	
Treatment delay (<i>days</i>)			
n=	23	22	
mean	9.17	9	p=0.72 ²
median	9	8	
range	1-26	1-22	

¹t-test

²t-test on log transformed values

Table 6.3: Virological response after initiating antiviral therapy according treatment allocation

Characteristics	Treatment allocation		Significance
	iv GCV	VGCV	
Time to PCR negative (days)			
n=	23	22	
mean	27.6	20.2	p=0.88 ¹
median	14	15.2	
range	2-230	0.-110	
Decline rate ($gen \times ml^{-1} \times day^{-1}$)			
n=	20	21	
mean	-0.57	-0.39	p=0.51 ¹
median	-0.4	-0.32	
range	-0.38 – -0.06	-0.92 – -0.02	
Day 7 log₁₀ decline ($gen \times ml^{-1}$)			
n=	18	20	
mean	-0.58	-0.94	p=0.09 ²
median	-0.64	-1.06	
range	-1.79 – -0.57	-1.96 – -0.075	
Day 14 log₁₀ decline ($gen \times ml^{-1}$)			
n=	12	10	
mean	-0.84	-0.88	p=0.92 ²
median	-1.16	-0.98	
range	-3.06 – -1.07	-2.12 – -0.29	
Treatment length (days)			
n=	20	21	
mean	17.7	23.9	p=0.12 ¹
median	16	20	
range	1-33	6-49	

¹t-test

²t-test on log transformed values

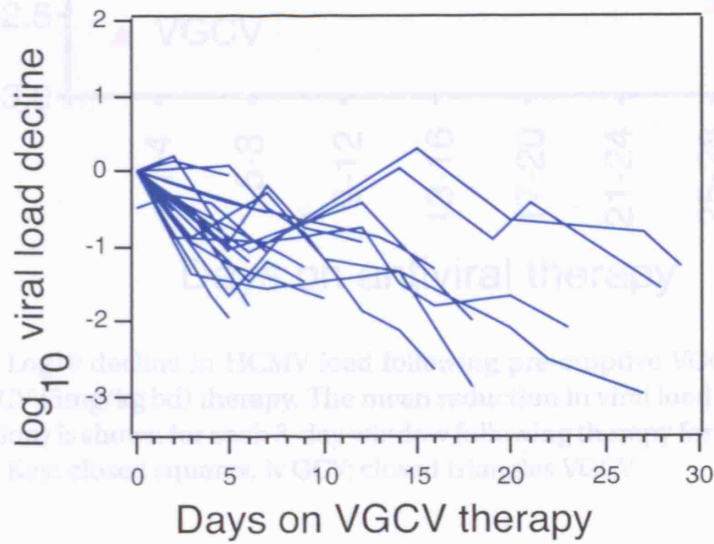
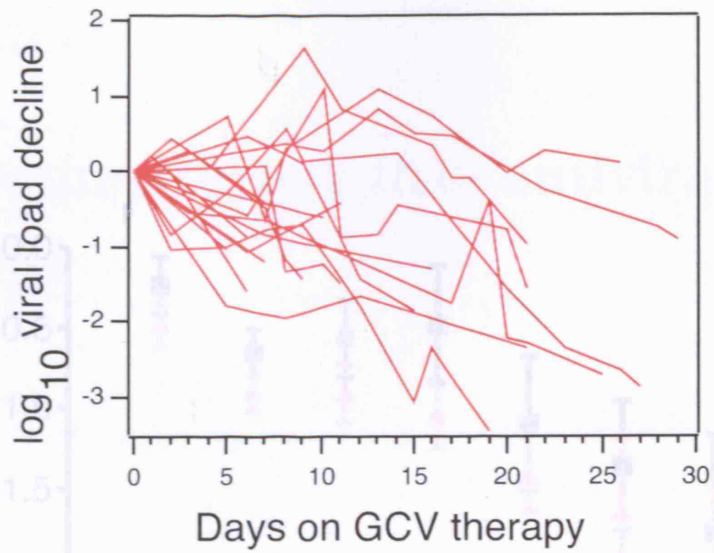


Figure 6.1: Decline of HCMV virus load from baseline after initiating antiviral therapy for individual patients receiving iv GCV (top panel) or oral VGCV (lower panel). In this analysis, change in virus load from baseline is plotted against days on antiviral therapy

Chapter 7

Predicting the length of antiviral therapy

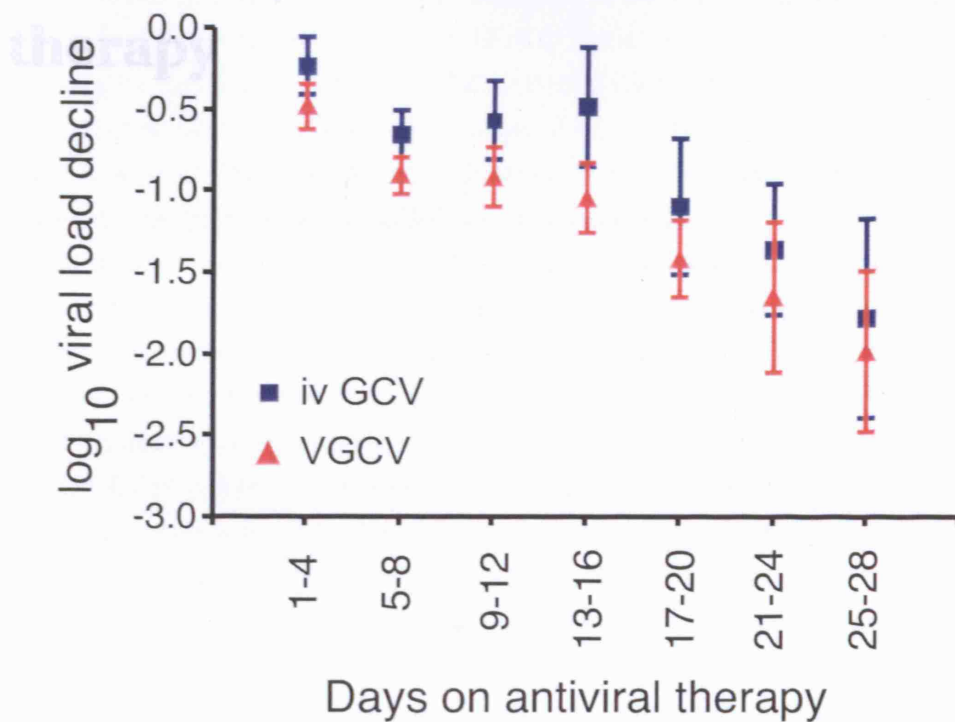


Figure 6.2: Log₁₀ decline in HCMV load following pre-emptive VGCV (900 mg bid) or iv GCV (5mg/kg bd) therapy. The mean reduction in viral load (+/- 1 standard deviation) is shown for each 3-day window following therapy for the 28 days on therapy. Key: closed squares, iv GCV; closed triangles VGCV

Chapter 7

Predicting the length of antiviral therapy

7.1 Introduction

Human cytomegalovirus (HCMV) infection remains a major risk factor for long term morbidity post solid organ transplantation [239]. However, mortality due to HCMV organ disease has been substantially reduced by anti HCMV prophylaxis or preemptive antiviral therapy.

Previous studies from others and our institution have identified several risk factors associated with HCMV disease, such as HCMV donor/ recipient status, administration of steroids, peak virus load in blood and urine (maximum virus load), virus replication rate and the detection of HCMV IgM antibodies in blood post transplantation [40, 41, 77, 111, 65, 63]. Donor/Recipient serostatus and the use of anti-thymocyte globulins (ATG) increase the virus load [111], whereas administration of methylprednisolone allowed HCMV disease to develop at a lower virus load [40]. Interestingly, in multivariable models, maximum virus load remains the only predictor for HCMV organ disease [63].

With close HCMV surveillance by blood PCR, HCMV disease has been minimized and is therefore an unsuitable marker for clinical studies. However, the length of viremia after starting antiviral therapy can be used as a marker of how well the host immune system together with antiviral therapy can control HCMV virus replication. For example a randomized clinical trial, comparing iv GCV versus iv GCV plus foscarnet has shown that higher baseline virus load and a faster replication rate is associated with the requirement of prolonged antiviral therapy in solid and bone marrow transplant patients (see chapter 5).

Monitoring HCMV load when on antiviral therapy becomes more difficult since most patients can be managed as outpatients, when on preemptive therapy with valganciclovir (see chapter 6). In addition, some patients might not benefit from preemptive therapy, because their preexisting immunity is able to control HCMV viremia without intervention.

The objective of this chapter was to identify factors which can predict the length of viremia after initiating antiviral therapy in the HCMV immune patient group, in particular factors which might be available before, starting antiviral therapy.

7.2 Methods

7.2.1 Patient selection

Patients with more than two consecutive positive HCMV PCR samples who received anti HCMV therapy were included in this study. The selected patients were identical to the cohort analyzed in chapter 6 (see table 6.1, page 157). In brief, the group consisted of 45 patients (27 liver, 18 renal, 23 male, 22 female), transplanted between October 2001 and September 2003 at the Royal Free Hospital. Patients received either iv GCV (5mg/kg bd) or VGCV (900mg bd), at the discretion of the attending physician. Antiviral therapy was initiated after the second positive HCMV PCR sample and continued until two consecutive negative PCR specimens. Patients with more than 30 days antiviral therapy or primary HCMV infection (donor HCMV seropositive, recipient HCMV seronegative) were excluded from the regression models, but included in the analysis of the cut-off viral load level to become HCMV PCR negative within 14 days. Only the first viremic episode which was treated was analyzed.

7.2.2 Statistical analysis

Scatterdiagrams were used to identify virological markers which predicted the length of antiviral therapy required to become HCMV PCR negative. Statistical significance was analyzed by simple linear regression and the quality assessed by the r^2 value of the linear curve fit.

Area under the viral load curve (AUC) was calculated with the trapezoidal rule on raw (not \log_{10} transformed) viral load data. Individual AUC values were divided by the days until start of antiviral therapy or time to become negative and then \log_{10} transformed.

Receiver operating curve (ROC) for baseline virus load (VL_{base}) and maximum virus load (VL_{max}) were used to calculate a threshold for becoming HCMV PCR negative within 14 days of therapy.

7.2.3 Definitions

The time (in days) to become HCMV PCR negative was defined as the interval between initiating antiviral therapy and the first negative PCR sample (<200 genomes/ml) followed by at least a second negative sample.

Baseline viral load was defined as the virus load at time of initiating antiviral therapy (+/- two days).

Maximum virus load was defined as the peak value observed during a viremic episode. Maximum virus load was stratified according the start of antiviral therapy, giving three groups. Maximum virus load before starting ($VL_{max_{before}}$), identical with baseline virus load ($VL_{max_{at}}$) or after starting antiviral therapy ($VL_{max_{after}}$).

Treatment delay was defined as the time interval from the first positive PCR sample until antiviral therapy begun. Viral loads and area under the viral load curve per day (AUC/d) are given in \log_{10} transformed values.

7.2.3.1 Calculation of viral replication and decline rate

A linear curve fit through all \log_{10} transformed viral load data, before and after starting antiviral therapy was used to calculate the replication rate (ρ) and decline rate (κ) as described in section 2.10.1.

7.3 Results

7.3.1 Maximum virus load in relation to start of antiviral therapy

Baseline virus load was available on 38 of the 45 patients, and ranged from 2.43 to 5.14 \log_{10} genomes/mL (median 3.54). Maximum virus load were included in the analysis from 39 patients (median: 3.87 \log_{10} genomes/mL, range: 2.92 to 5.74 \log_{10} genomes/mL), excluding 6 patients with primary HCMV infection or longer than 30 days time to become HCMV PCR negative. Median time to become HCMV PCR negative was 13 days (range 2 to 28 days). Sixteen patients had their maximum virus load before, 14 at the time and 14 after initiating antiviral therapy.

A very good correlation between baseline virus load and maximum virus load was found and was independent of the timing of maximum virus load relative to start of antiviral therapy (figure 7.1, page 173, see different colour labels in the scatter diagrams).

Patients with an increasing virus load after initiating antiviral therapy took a longer time to become HCMV PCR negative compared to patients with the max-

imum virus load before or at the time of initiating antiviral therapy (median time to PCR negative 20 days, for patients with maximum virus load after initiating therapy, 17 days for maximum virus load at time of initiating therapy, 6 days if maximum virus load before starting therapy, ANOVA $p=0.0026$). Maximum HCMV loads were higher in patients with a maximum virus load after initiating therapy (VL_{max} : 4.91_{after}, 4.21_{at}, 3.54_{before}, ANOVA $p=0.003$). However, replication rate ($VL_{max_{after}}$ 0.34 day⁻¹, $VL_{max_{at}}$ 0.32 day⁻¹, $VL_{max_{before}}$ 0.43 day⁻¹, ANOVA $p=0.29$) and treatment delay (5 days_{after}, 10 days_{at}, 7 days_{before}, ANOVA $p=0.29$) was not significantly different between the groups.

7.3.2 Baseline and maximum virus load correlates with time to become PCR negative

Scatterplots of different viral kinetic markers and time to become PCR negative were derived and if appropriate, simple linear regression analysis performed. Only baseline virus load (see figure 7.2, page 174) and maximum virus load (figure 7.3, page 175) were predictive of time to become PCR negative. No difference for patients with maximum virus load before or after initiating antiviral therapy were observed (see different color labels in the scatter diagrams).

7.3.3 Area under the curve (AUC) before antiviral therapy as a predictor length of viremia

Because viral replication rate was not a predictor for time to become PCR negative ($R^2=0.1$) the log transformed viral load area under the curve per day ($\log_{10}(\text{AUC}/\text{day})$ [genomes/mL]) was investigated.

The $\log_{10}(\text{AUC}/\text{day})$ did not predict the time to become HCMV PCR negative directly (figure 7.4, page 176), but $\log_{10}(\text{AUC}/\text{day})$ before starting antiviral therapy correlates with $\log_{10}(\text{AUC}/\text{day})$ after starting therapy (figure 7.5, page 177).

In addition, the total area under the viral load curve before starting pre-emptive therapy was not different from the area under the viral load curve until HCMV PCR negative in immune experienced patients (paired t-test: $p=0.11$).

$\log_{10}(\text{AUC}/\text{day})$ before therapy correlated with the achieved baseline virus load (figure 7.6, page 178) and maximum virus load (figure 7.7, page 179). There was no difference in correlation for patients who had the peak virus load before or after starting therapy (see different color code in figure 7.6, page 178 and 7.7,

page 179).

7.3.4 Defining diagnostic virus load levels for becoming HCMV PCR negative by 14 days

Because baseline virus load and maximum virus load correlate well with the time to become PCR negative, the possibility of defining a virus load threshold for becoming HCMV PCR negative within 14 days of therapy was investigated. Baseline virus load and maximum virus load were each statistically significantly lower in patients who cleared viremia in blood within 14 days of therapy. Median baseline virus load was 3.21 log₁₀genomes/mL (range: 2.29-3.99) for patients who became negative within 14 days compared to 4.35 log₁₀genomes/mL (range: 2.92-5.75) who required longer therapy (Mann-Whitney U test p<0.001, figure 7.8, page 180 top panel). Similarly, median maximum virus load 3.47 log₁₀genomes/mL (range: 2.80-4.60) for patients who became negative within 14 days compared to patients who required longer antiviral therapy (median: 4.91 log₁₀genomes/mL, range: 3.59-5.82, figure 7.8, page 180 lower panel).

A receiver operating curve was constructed for baseline virus load and maximum virus load, with the data stratified into time to become HCMV PCR negative less than 14 days versus more than 14 day. The ROC graphs are shown in figure 7.9, page 181. For both virological markers, 4 log₁₀genomes/mL was identified as a threshold which gave the highest sensitivity and specificity (VL_{base}: sensitivity 95.45%, specificity 61.54%; VL_{max}: sensitivity 95.65%, specificity 92.31%; see table 7.1 at page 182).

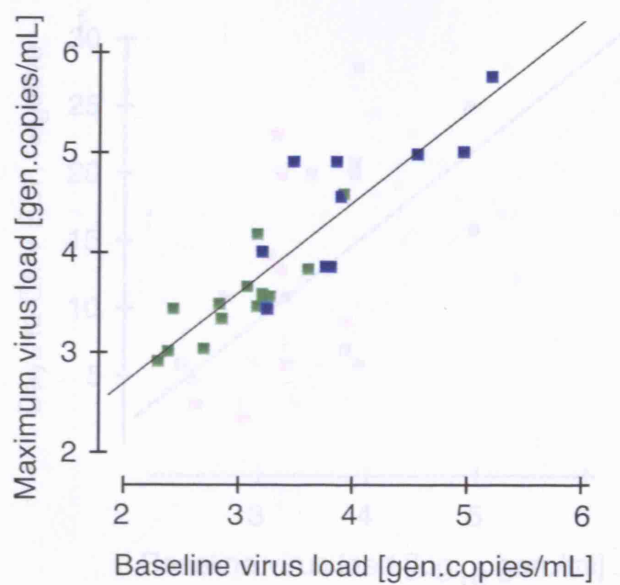


Figure 7.1: Scatterdiagram showing the correlation baseline HCMV virus load and maximum virus load. Green labels indicating patients with peak virus load before starting antiviral therapy, blue labels patients with peak virus load after initiating antiviral therapy. Patients whose baseline virus loads were identical have been excluded ($r^2=0.79$).

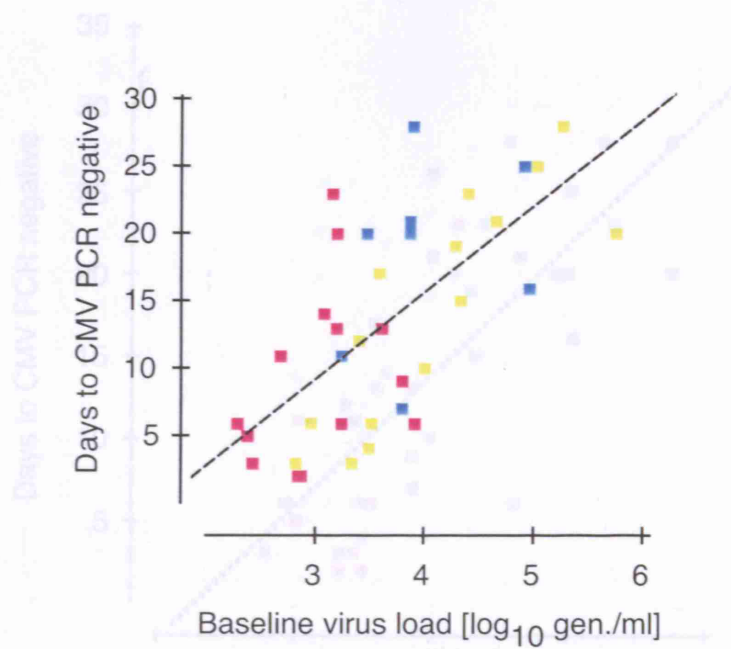


Figure 7.2: Scatterdiagram showing the correlation between baseline HCMV virus load and time to become HCMV PCR negative. Yellow labels indicate patients with peak virus load before starting antiviral therapy, red labels; patients with peak virus load at day of starting antiviral therapy; blue labels patients with peak virus load after initiating antiviral therapy ($r^2=0.44$).

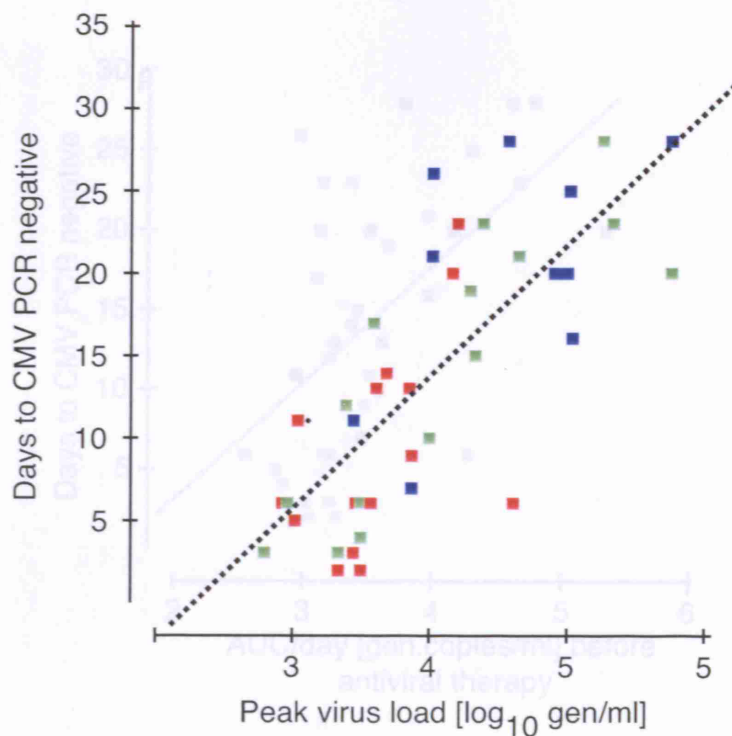


Figure 7.3: Scatterdiagram showing the correlation between peak virus load and time to become HCMV PCR negative. Green labels indicate patients with peak virus load before starting antiviral therapy, red labels; patients with peak virus load at day of starting antiviral therapy; blue labels patients with peak virus load after initiating antiviral therapy ($r^2=0.58$).

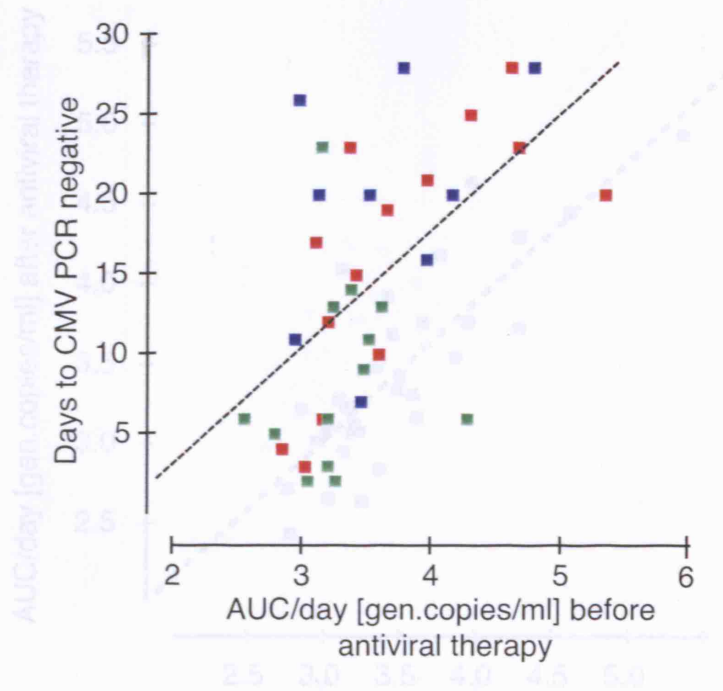


Figure 7.4: Scatterplot showing the relationship between AUC/day before antiviral therapy and time to become HCMV PCR negative. Green, patients with peak virus before therapy start; red labels patient with peak virus at antiviral start and blue labels patients with peak virus load after starting therapy ($r^2=0.29$).

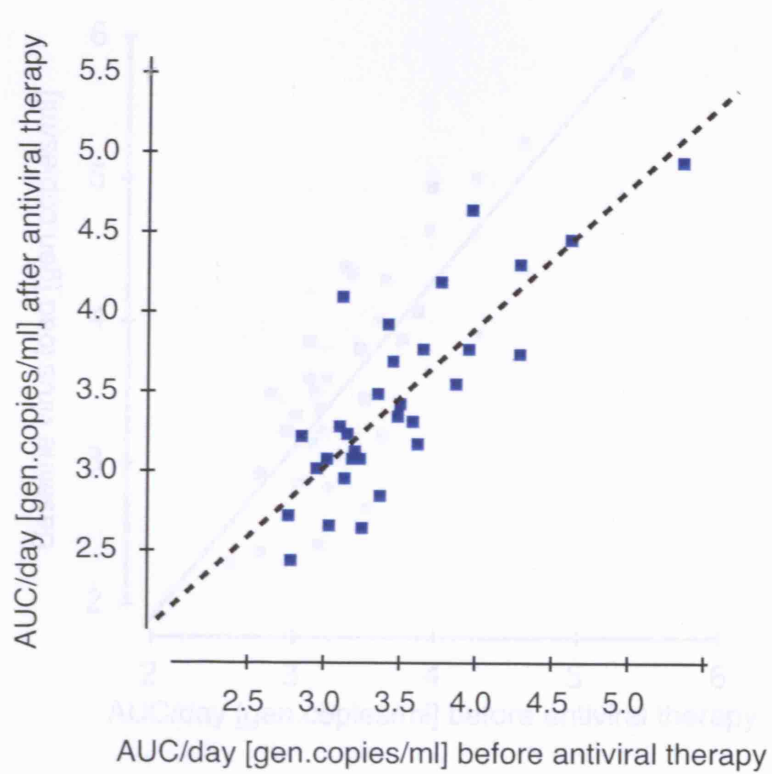


Figure 7.5: Scatterplot showing the relationship between area under the viral load curve per day (AUC/d, log₁₀ transformed) before initiating antiviral therapy and AUC/d after starting therapy, until becoming HCMV PCR negative ($r^2=0.65$).

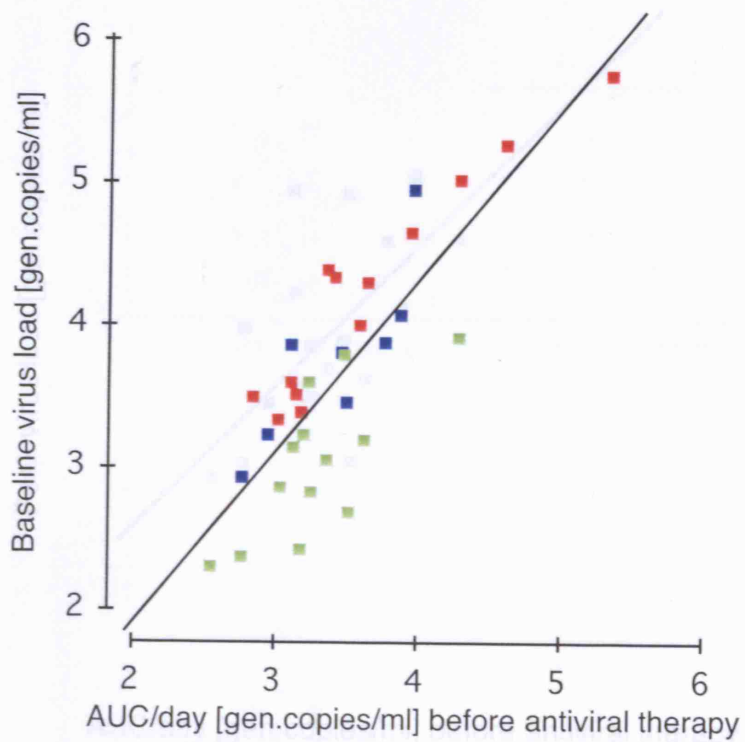


Figure 7.6: Scatterplot showing the relationship between AUC/day before antiviral therapy and baseline virus load. Green, patients with peak virus before therapy start; red labels patient with peak virus at antiviral start and blue labels patients with peak virus load after starting therapy. AUC/d \log_{10} transformed ($r^2=0.67$).

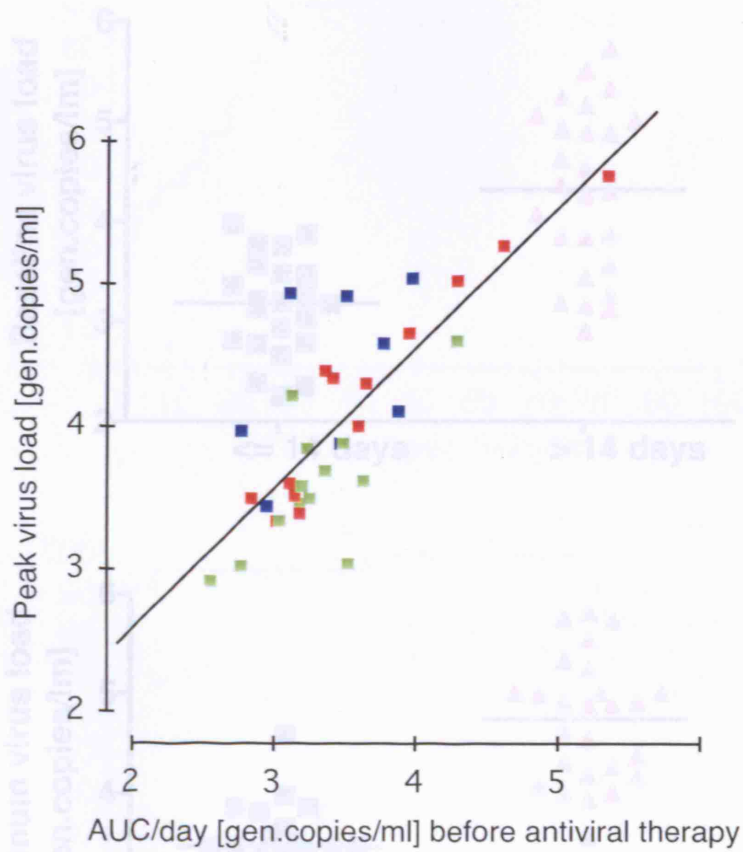


Figure 7.7: Scatterplot showing the relationship between AUC/day before antiviral therapy and peak virus load. Green, patients with peak virus before therapy start; red labels patients with peak virus at antiviral start and blue labels patients with peak virus load after starting therapy ($r^2=0.66$).

Figure 7.8: Dotplot comparing baseline virus load (top panel) and maximum virus load (lower panel). Data are stratified to patients with less and more than 14 days antiviral therapy.

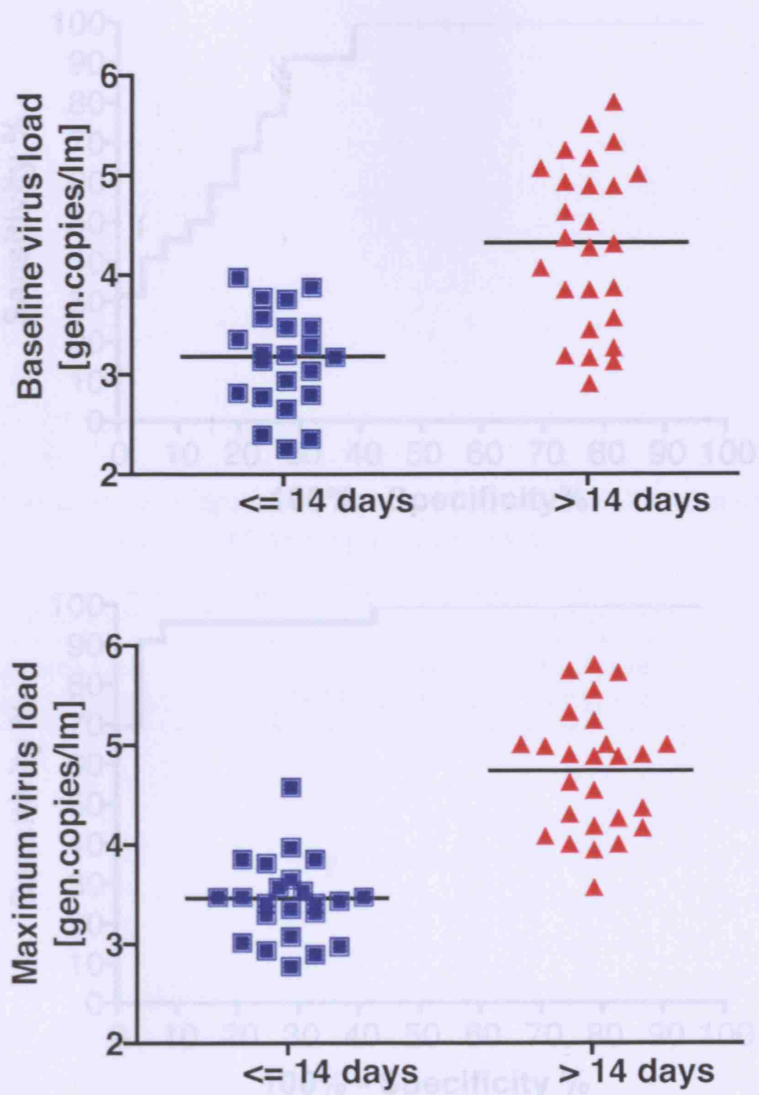


Figure 7.8: Dotplot comparing baseline virus load (top panel) and maximum virus load (lower panel). Data are stratified in patients with less and more than 14 days antiviral therapy.

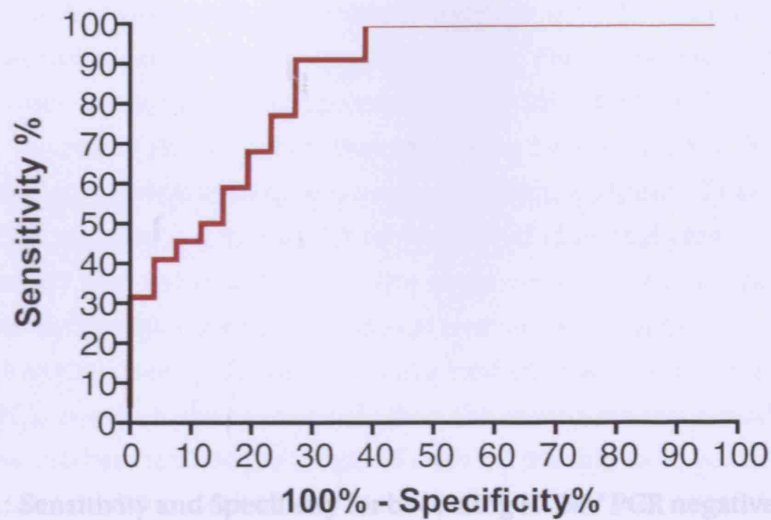


Table 2.1: Sensitivity and Specificity for becoming HCMV PCR negative within 14 days with a threshold of 10000 genomes copies per mL.

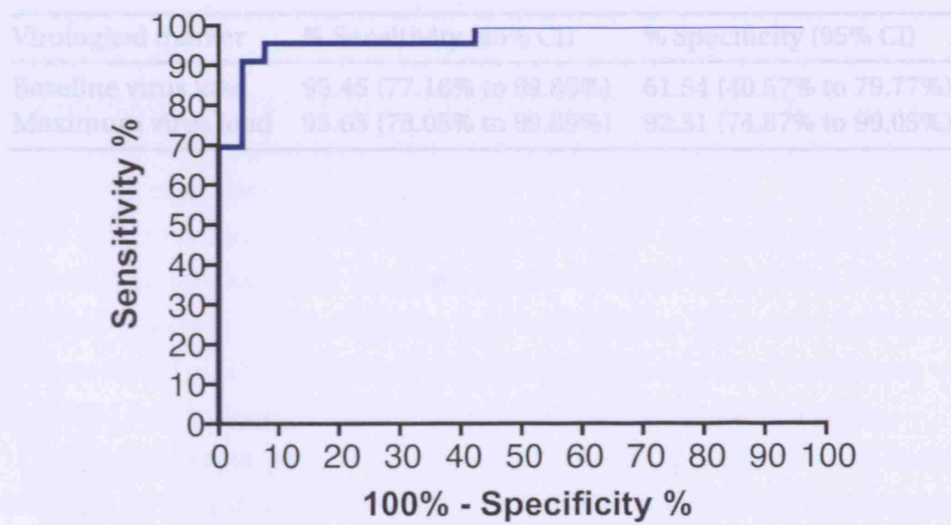


Figure 7.9: Receiver operating characteristics (ROC) for becoming HCMV PCR negative within 14 days for baseline virus load (top panel) and maximum virus load (lower panel)

Table 7.1: Sensitivity and Specificity for becoming HCMV PCR negative within 14 days with a threshold of 10000 genomes copies per mL

Virological marker	% Sensitivity (95% CI)	% Specificity (95% CI)
Baseline virus load	95.45 (77.16% to 99.88%)	61.54 (40.57% to 79.77%)
Maximum virus load	95.65 (78.05% to 99.89%)	92.31 (74.87% to 99.05%)

7.4 Discussion

Previous studies have identified peak virus load and replication rate as major predictors for HCMV organ disease in solid organ transplant patients [65]. Alternatively, the first virus load measurement together with the replication rate can predict the likelihood of HCMV organ disease [65]. This chapter extends this concept to patients who were preemptively treated with iv GCV or VGCV to prevent disease. Instead of HCMV organ disease, time to become HCMV PCR negative after initiating antiviral therapy was used as a clinical endpoint. Time to become HCMV PCR negative is a marker for the control of virus replication by preexisting immunity plus antiviral therapy. The study showed that baseline virus load correlates highly with maximum virus load (see figure 7.1, page 173).

Baseline virus load and maximum virus load correlated with time to become HCMV PCR negative, irrespective of when the maximum virus load occurred. These two markers allowed the length of antiviral therapy to be predicted.

The AUC over the virus load per day before starting antiviral therapy was identical to the AUC per day until PCR negative in immune experienced patients (see figure 7.10 at page 185, panel A). In contrast, AUC/d after starting therapy is larger in patients with primary HCMV infection (panel B). The difference in the AUC/d after therapy between these two groups (shaded area in figure 7.10, panel C) may well be the effect of the preexisting immunity, but further studies are required to assess this possibility.

No direct marker was identified, which could be measured, before starting antiviral therapy and could reliably predict the time to become HCMV PCR negative. However, maximum virus load and baseline virus load were predictors based on the AUC/d before starting therapy. The calculated maximum virus load could subsequently be used to determine the time to PCR negative.

In our institution with frequent sampling for HCMV surveillance, baseline virus load is normally available. With the introduction of VGCV for preemptive therapy, many patients are now managed as outpatients, and follow up to monitor decline of virus load becomes more difficult. In this chapter I showed that a threshold of $4 \log_{10}$ genomes/mL differentiates patients who become negative for HCMV in blood within 14 days. This finding could potentially be translated into patient management. Patients with more than $4 \log_{10}$ genomes/mL can be sent home with a supply of tablets for at least 14 days, with a virus load measure at day 7 to confirm a decline of virus load. Vice versa, patients with a low virus

load might need only a short 14 day course of therapy. A clinical trial would be required to assess the usefulness of such a change in patient management.

The results showed also, that maximum or baseline virus load is not only a predictor for HCMV organ disease [65], but also a predictor for the length of antiviral therapy required to become HCMV PCR negative.

In contrast, this study could not confirm previous finding, that replication rate prior to antiviral is a predictor for maximum virus load or baseline virus load [65]. This may be explained by different study outcome (HCMV organ disease vs length of antiviral therapy), by the smaller number of patients included in this study, exclusion of patients with primary HCMV infection and the prompt antiviral therapy after detecting HCMV in blood (median treatment delay 9 days, between first positive PCR sample and initiating antiviral therapy). In addition this study was restricted to solid organ transplant patients who became HCMV negative within 30 days.

Several questions remain unanswered and require a further study. Is it possible to predict maximum virus load or baseline virus load by calculation of the AUC/d based on interval between the last negative to first positive PCR sample? If so, how accurately can a predicted maximum virus load predict further the time to PCR negative and is the error in prediction still acceptable in clinical practice? Furthermore, can the measurement of the cellular immunefunction and functional capacity as described in chapter 4 help to improve the prediction. Prospective clinical trials would be required to assess these concept.

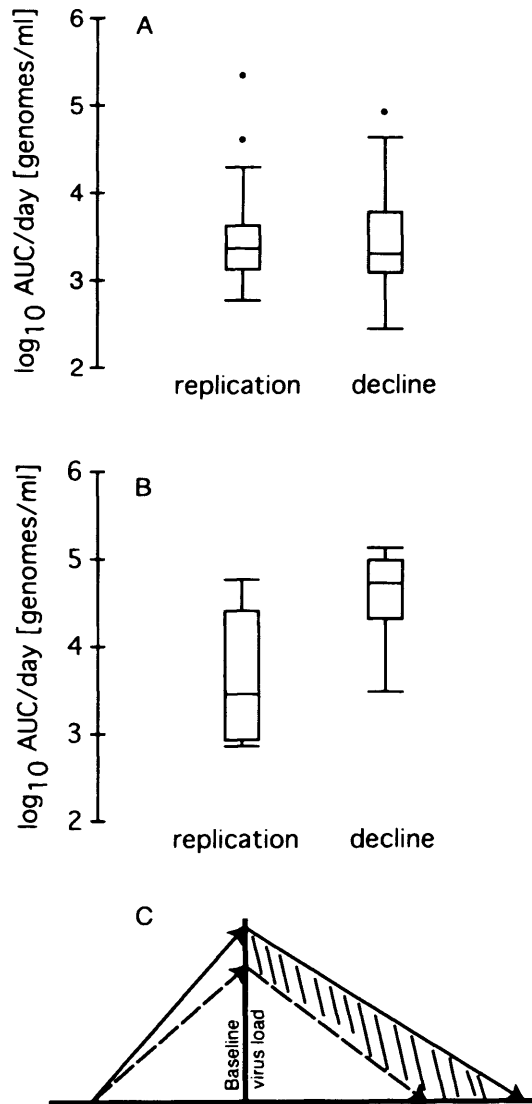


Figure 7.10: Panel A: Boxplot showing \log_{10} AUC/d before starting antiviral therapy compared to \log_{10} AUC/d until becoming HCMV PCR negative in immune experienced patients (paired t-test $p=0.46$). Panel B: Boxplot showing \log_{10} AUC/d before starting antiviral therapy compared to \log_{10} AUC/d until becoming HCMV PCR negative in patients without pre existing immunity (primary HCMV infection, panel B, paired t-test $p=0.03$). Panel C: Graphical illustration how pre existing immunity influences area under the viral load curve before and after initiating therapy.

Chapter 8

General Discussion

Human cytomegalovirus remains an important pathogen in immunocompromised patients, such as HIV infected patients solid and bone marrow transplant patients [100]. In my thesis I addressed several current questions concerning the management of these patients.

8.1 Diagnosis of HCMV end-organ disease

Cytomegalovirus is a common opportunistic pathogen which infected with often results in end-organ diseases such as retinitis, colitis, pneumonitis [297] and encephalitis. HCMV end-organ disease is routinely diagnosed by histological examination of organ biopsies and the demonstration of intranuclear inclusion bodies (owl's eye inclusion bodies) which are considered to be diagnostic for HCMV [169, 164]. However human herpes virus 6 (HHV 6) [245] and human herpes virus 7 (HHV 7) [245, 15], two other members of the betaherpesvirinae family, may cause end organ disease similar to HCMV in immunocompromised patients and it is not known if these can also produce owl's eye inclusions.

In chapter 3 of my thesis I investigated if HHV 6 or HHV 7 can cause owl's eye inclusions. I found a significant association between positive HCMV PCR and the detection of owl's eye inclusion bodies in organs (table 3.1, page 101). No association was detected between positive HHV 6 or HHV 7 tissue PCR and owl's eye inclusions (figure 3.2 and 3.3, page 101). In addition the virus load for HCMV was higher in organs with detection of owl's eye inclusions, but no difference in virus load found for HHV 6 or HHV 7 in organs with or without owl's eye inclusion bodies was seen (figure 3.1, page 102).

This study showed that the histological finding of owl's eye in tissue or organ biopsy samples remains highly specific for HCMV. However this study showed also, that not finding owl's eye inclusions does not exclude HCMV organ disease, due to the low sensitivity, so that more sensitive assays should be used, such as immunohistochemistry, in situ hybridization or HCMV PCR of tissue DNA extracts.

8.2 Treatment with ganciclovir

HCMV end-organ disease is associated with a high morbidity and mortality in immunocompromised patients and requires a prolonged antiviral therapy [99].

Several strategies have been developed to prevent HCMV organ disease, such as prophylaxis or pre-emptive therapy [240, 60, 110]. Combined with close monitoring of HCMV infection in the blood by PCR, HCMV end organ disease has now become a rare clinical entity. Consequently end-organ disease can not longer be used as a clinical marker to study HCMV pathogenesis. In my thesis I examined whether, HCMV viral kinetic markers could be used as study endpoints. I also asked if viral replication kinetics or the length of antiviral therapy required to become HCMV PCR negative can be used as a surrogate marker of how effective the immune system is at controlling HCMV replication or how effective the intervention with a antiviral therapy is.

Intravenous GCV (iv GCV) was frequently used in the past as preemptive therapy for HCMV infection. These patients are often clinically well, but cannot be discharged because of the difficulties associated with the administration of an intravenous drug. Alternatively, if they are outpatients, they have to come back into hospital. Recently, the prodrug valganciclovir was developed, which has similar plasma levels to iv GCV [134, 27]. No data have been published, reporting the experience with Valganciclovir in solid organ transplant recipients in a preemptive therapy setting for HCMV infection.

Valganciclovir was compared against iv GCV in a non randomized clinical study (chapter 6). The primary endpoint of the study was defined as time required to become HCMV PCR negative in blood, the \log_{10} virus load decline from baseline at day 7 and day 14 and the decline rate after initiating antiviral therapy. Of the 45 patients, 23 received iv GCV and 22 VGCV. Both arms were well matched in terms of important demographic and virological parameter (table 6.1 page 162 and 6.2 page 163). Furthermore no difference was observed in the type of immunosuppressive drug regime used.

No difference between the two treatment arms was found for the time to become HCMV PCR negative. In addition, no difference in the decline rate, \log_{10} decline from baseline virus load at day 7 and 14 was observed. However, in the iv GCV arm, I noticed an increase in virus load in some patients which was unexpected, and not found in the VGCV arm (figure 6.1, page 165). These patients were identified as those with a primary HCMV infection (D+/R-), and were more frequently in the iv GCV treatment arm, although this difference was not statistically significant. Recently a study reported similar rates for preventing HCMV disease for 6 and 12 months in the D+/R- solid organ transplant patient group when compared to oral GCV [201]. In addition, valganciclovir was better at pre-

venting HCMV viremia. The better oral bioavailability of Valganciclovir is reflected by the finding of a significant lower resistance against GCV in D+R- solid organ patient group receiving either oral GCV or VGCV as prophylaxis [19].

8.3 Treatment with foscarnet/ganciclovir combination

Treatment with GCV causes severe bone marrow suppression which requires discontinuation, or switching to foscarnet [229]. However, one of the major side effects associated with foscarnet is renal toxicity, which makes it unsuitable in renal transplant patient or in patients with pre-existing renal impairment. In vitro data and a randomized clinical trial for the treatment of retinitis in HIV infected patients suggested a synergistic effect between the two antiviral drugs [107, 172]. This led to the implementation of a randomized clinical trial in BMT, Liver and Renal transplant patients, comparing the full dose of iv GCV versus iv GCV plus foscarnet at half dose each in the preemptive therapy for HCMV infection (chapter 5). The primary study endpoint was the proportion of patients becoming HCMV PCR negative within 14 days. Secondary study endpoints was defined as the number of patients with side effects associated with the study drug. The hypothesis was that the reduced dose of GCV and foscarnet would give better antiviral control of HCMV with a reduced number of patients with drug related side effects.

No statistical significantly difference in the proportion of patients who became HCMV PCR negative within 14 days was found, with a trend in favour of GCV (see table 5.2, page 140; figure 5.1, page 141). In addition, all seven patients who experienced drug toxicity were allocated to the combination arm, and the side effects in 6 of the 7 patients were most likely attributable to use of foscarnet (increase in creatinine and electrolyte imbalance; table 5.3, page 143).

8.4 Identifying patients who fail therapy

Before the introduction of valganciclovir into clinical practice, most patients required hospitalization for the duration of the antiviral therapy. Hence monitoring of HCMV load while on therapy, can be easily arranged. However, since the licensing of valganciclovir, many patients can be discharged home so obtaining frequent blood samples on these patients for monitoring the decline of virus load

is more difficult to organize. A virological marker, easy to measure at the beginning of therapy which can accurately predict the minimum length of antiviral therapy would be useful.

More detailed analysis of the viral load data and calculation of replication and decline rates, identified risk factors associated with not reaching the primary endpoint (faster replication rate, slower decline rate, higher baseline virus load; table 5.4, 151). Virological follow up of the study patients identified the patient group, who failed in the primary endpoint, as more likely to reactivate HCMV again within the next 100 days (table 5.6 and table 5.7, page 154).

High virus load at baseline was identified as a risk factor for not becoming PCR negative within 14 days of therapy (chapter 5). In addition a linear correlation between baseline virus load and decline rate was observed after stratifying the patients according to the primary study endpoint (figure 5.5, page 148). It is also possible that HHV-6 and HHV-7 co-infections may contribute to poor response rate of HCMV. However, data suggest that inhibition of HHV-6 replication in vivo with GCV plus foscarnet is comparable to that observed for HCMV [131].

In chapter 7, I investigated the possibility to identify markers which predicted the length of antiviral therapy required, using the data from liver and renal transplant patients threatened with either iv GCV or valganciclovir (see chapter 6). Baseline virus load predicted maximum virus load (figure 7.1, page 173), and baseline and maximum virus load could be used to predict the time to become HCMV PCR negative (figure 7.2, 174; figure 7.3, page 175). However, baseline virus load is unknown until the start of therapy, and maximum virus load can only be defined after the patients became HCMV PCR negative. This study showed also, that the area under virus load time curve (AUC) is not only a predictor for subsequent HCMV disease [248], but also correlates with the maximum virus load and baseline virus load (page 7.6, page 178; figure 7.7, page 179). The study was able to identify a single marker which can be measured on the first or second positive sample which predicted the length of antiviral therapy. However, measuring the AUC/d, based on the last negative HCMV PCR sample and the first two positive HCMV PCR samples might be sufficiently accurate to predict the maximum virus load, which could be used to predict the length of antiviral therapy. This analysis was not possible in this cohort, because of the prompt initiation of antiviral therapy after the second positive sample (treatment delay < 9 days).

The study was able to demonstrate that a cut off level of 10000 genomes/mL discriminated patients who require more than 14 days therapy. In the immune

experienced patient group AUC/d before starting therapy is similar to the AUC/d after starting therapy, whereas AUC/d after therapy start is larger than AUC/d before starting therapy in the patient group with a primary HCMV infection (figure 7.10, page 185, panel C). The difference between the AUC/d of the two groups might reflect a contribution of preexisting immunity and this could be studied in the future. Predicting whether a patient requires 7, 14, 21 days or longer antiviral therapy will be useful, to arrange clinic visits and individualize the antiviral therapy. It is possible that some of these patients may also be experiencing HHV-6 and 7 infection, although previous data showed that the virus load is significantly lower for these viruses compared to HCMV [146, 96]. Nevertheless, coinfection could impair the response of HCMV to therapy and should be considered in future trials investigating the response of HCMV to therapy.

8.5 Markers of cellular immunity

The cellular immune response (CD8+ T cells) against HCMV is mainly directed against the lower matrix protein pp65 (UL83) [300] and is a key element in controlling HCMV virus replication during the primary infection or reactivation. Several HLA class I restricted immunodominant proteins have been identified [300, 81, 176]. Tetrameric complexes can be used to enumerate virus specific CD8+ T-cells *ex-vivo* without culturing the cells [159]. In combination with a functional assay (ELISpot, intracellular cytokine staining) the frequency and functional capacity of virus specific CD8+ T cells can be assessed. Previous studies have shown, that a large proportion of CD8+ T cells are directed against a single viral peptide in healthy individuals and immunocompromised patients [79, 113, 9, 67]. In addition, in healthy blood donors, an accumulation of virus specific HCMV tetrameric complex positive cells occurs [138].

HCMV viremia was used as a surrogate marker of how well the cellular immune system is able to control HCMV virus replication post renal transplantation (Chapter 4, page 108). The 10 patients who became viremic (8/10 immunexperienced) had higher virus specific CD8+ T-cells (measured by tetrameric complexes), than the 15 patients who remained HCMV PCR negative, and the difference was present before becoming viremic (figure 4.5, page 121 and table 4.6, page 130). This finding led me to reject the hypothesis that virus specific CD8+ T-cells measured by tetrameric complexes are protective. Functional analysis with the ELISpot assay also showed no difference in the functional capacity between

viremic and non viremic patients (figure 4.6, page 122 and table 4.7, page 130). However, further analysis of the pp65 tetrameric positive CD8+ T-cells showed a difference in the functional capacity between viremic and non viremic patients. In contrast to viremic patients, in which only 20% of the tetrameric positive CD8+ T-cells are able to secrete IFN- γ , 80% of these cells are able to respond with IFN- γ secretion in non viremic patients (figure 4.9, page 125). The proportion of IFN- γ secreting cells within the pp65 specific (tetrameric+) CD8+ cell population is the dominant predictor for viremia in the future (figure 4.3.7, page 126; univariable models 4.5, page 129). The difference cannot be explained by a difference in the immunosuppressive drug regime, which was very similar within both groups.(table 4.3, page 128). A similar finding was observed in liver transplant patients.

This functional defect may precede renal transplantation and might well be present in haemodialysis patients. Blood samples collected from haemodialysis patients are now being tested to see if this immune dysfunction can be seen already in this patient group. Moreover, routine enumeration of HCMV specific pp65 specific CD8+ T-cells with tetrameric complexes together with a functional ELISpot assay soon after transplantation might allow identification of patients at high risk of viremia (eg low capacity of secretion of IFN- γ) and this is another hypothesis which could be tested in the future.

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Appendices

Appendix A

List of manufacturers

Alpha Laboratories	Hampshire SO50 4NU, UK
Amersham Biosciences	Bucks HP8 4SP, UK
Amicon	Millipore Watford, WD18 8YH, UK
BD Biosciences	Oxford OX4 3LY, UK
BDH Laboratories Supplies	Lutterworth LE17 4XN, UK
Bio-Rad Laboratories	Hemel Hempstead HP2 7TD, UK
Dako	Ely CB7 4ET, UK
Falcon	Oxford OX4 3LY, UK
GMI	Albertville, Minnesota, USA
Hybaid	Ashford, TW15 1XB UK
Invitrogen	Paisley, UK
Mabtech	28 Nacka Strand, Sweden
Millipore	Millipore Watford, WD18 8YH, UK
Novagen	Merck Biosciences, Nottingham, NG9 2JR
Nulgene	Rochester, NY, USA
Quiagen	Crawley, RH10 9NQ, UK
Sarstedt	Leicester LE4 1AW, UK
Sigma-Aldrich	Gillingham SP8 4XT, UK

Appendix B

Buffers and cell culture media

B.1 R-10 cell culture medium

RPMI-1640 450 ml
FCS 50 ml
Glutamin 5 ml

B.2 Media and plates for bacterial cultures

B.2.1 Luria-Bertani (LB) Medium

Tryptone 10g
NaCl 10g
Yeast extract 5g
add 1L H_2O , autoclave.

B.2.2 Luria-Bertani (LB) agar plates

Tryptone 10g
NaCl 10g
Yeast extract 5g
Agar 15g
add 1L H_2O , autoclave. LB agar plates

B.3 Buffers for tetrameric complex synthesis

B.3.1 Triton wash buffer

Triton X-100	0.5%
Tris pH 8	50mM
NaCl	100mM
EDTA	1mM
Na-Azide	0.1% (w/v)
DTT	1mM

B.3.2 Urea buffer

Urea	8M
NaH ₂ PO ₄	100mM
Tris pH 8	10mM
EDTA	0.1mM
DTT	0.1mM

add duolite indicator resin (BDH); use if blue.

B.3.3 Refolding buffer

Tris pH 8.0	100mM
L-Arginine*HCl	400mM
Glutathione (reduced)	5mM
Glutathione (oxidised)	0.5mM
EDTA	2mM

B.3.4 BirA buffer

Tris pH 8.0	20mM
NaCl	50mM
MgCl ₂	20mM

B.3.5 FPLC buffer

Tris pH 8.0	20mM
NaCl	50mM

B.3.6 MonoQ anion exchange buffer A and B

Buffer A

Tris pH 8.0 20mM

Buffer B

Tris pH 8.0 20mM

NaCl 1000mM

B.3.7 phosphate buffered saline (PBS, pH 7.4)

Na₂HPO₄ 0.61g

NaH₂PO₄ 0.1g

NaCl 8.7g

make up to 1L with distilled water and sterile filter.

B.4 Publications

Parts of thesis have been published

Chapter 3

Mattes FM, McLaughlin JE, Emery VC, Clark DA and Griffiths PD (2000). Histopathological detection of owl's eye inclusions is still specific for cytomegalovirus in the era of human herpesviruses 6 and 7. *Journal of Clinical Pathology*, 53: 612-614.

Chapter 5

FM Mattes, EG Hainsworth, AM Geretti, G Nebbia, HG Prentice, M Potter, AK Burroughs, P Sweny, AF Hassan-Walker, S Okwuadi, CA Sabin, G Amooty, VS Brown, SC Grace, VC Emery, PD Griffiths (2004). A randomized, controlled trial comparing ganciclovir or ganciclovir plus foscarnet (each at half dose) for pre-emptive therapy of cytomegalovirus infection in transplant recipients. *J. Infectious Disease*, 189:1355-1361.

Chapter 6

Mattes FM, Hainsworth EG, Hassan-Walker AF, Burroughs AK, Sweny P, Griffiths PD and Emery VC (2004) Kinetics of cytomegaloviral load decline in solid organ transplant recipients after pre-emptive valganciclovir therapy. *J. Infectious Disease*, (in press).

Histopathological detection of owl's eye inclusions is still specific for cytomegalovirus in the era of human herpesviruses 6 and 7

F M Mattes, J E McLaughlin, V C Emery, D A Clark, P D Griffiths

Abstract

Background—Cytomegalovirus (CMV) is the prototype member of the β -herpesvirinae, which can cause multiple organ dysfunction in the immunocompromised host. Human herpesvirus 6 (HHV-6) and HHV-7 are newer members of the β -herpesvirinae that can cause febrile illness in young children and are also possible pathogens in the immunocompromised patient.

Aim—CMV is detected in histopathological sections by visualisation of owl's eye inclusion bodies. The aim of this study was to quantify the relation between CMV, HHV-6, and HHV-7 viral loads and the presence of owl's eye inclusions in histological sections.

Methods—Histopathological examination of postmortem material and recording of owl's eye inclusion bodies were performed. CMV, HHV-6, and HHV-7 were detected by qualitative and quantitative polymerase chain reaction (PCR) from the same postmortem samples. Statistical analysis of the histopathological and PCR results was performed.

Results—There was a significant association between the detection of owl's eye inclusion bodies and positive CMV PCR ($p < 0.001$); the median CMV viral load was significantly higher in samples that were positive for owl's eye inclusions ($p < 0.001$). No association was found between the presence of owl's eye inclusions and HHV-6 or HHV-7 positivity.

Conclusion—Histological detection of owl's eye inclusion bodies is an insensitive but highly specific method for detecting CMV organ involvement. Owl's eye inclusion bodies are not associated with HHV-6 or HHV-7 infection.

(*J Clin Pathol* 2000;53:612-614)

Keywords: polymerase chain reaction; inclusion bodies; viral load

Cytomegalovirus (CMV) is an important cause of multiple organ dysfunction in the immunocompromised host.¹ Patients can present with hepatitis, pneumonitis, ulceration of the oesophagus or colon, retinitis, or encephalitis. Organ involvement is routinely diagnosed by biopsy, with visualisation of owl's eye intranuclear inclusions in stained tissue sections.^{2,3}

CMV (human herpesvirus 5) is the prototype member of the β -herpesvirinae, a subfamily of the herpesviridae.⁴ In 1986 and 1990,

respectively, two new herpesviruses were described and allocated to the β -herpesvirinae on the basis of their strong genetic relatedness to CMV; these viruses are termed human herpesvirus 6 (HHV-6)⁵ and HHV-7.^{6,7} HHV-6 and HHV-7 can each cause febrile illness in young children, including exanthem subitum,⁸⁻¹⁰ and case reports suggest that, like CMV, HHV-6 may cause end organ disease in the immunocompromised host.¹¹ Other reports suggest that CMV associated disease might be increased in patients co-infected with HHV-7^{12,13} or HHV-6.¹⁴ It is not known whether HHV-6 and/or HHV-7 can produce owl's eye inclusions in vivo but, if they do, this could complicate the interpretation of a postulated association between these other viruses and CMV associated disease.

We have developed quantitative competitive polymerase chain reaction (QCPCR) methods to detect each of these three β -herpesviruses¹⁵⁻¹⁷ and quantify the viral load in biological samples, including tissue specimens.¹⁶ In our study, we used these techniques to determine the sensitivity of histopathological visualisation of owl's eye inclusions to detect CMV infection and whether their presence is specific for CMV alone among the β -herpesvirinae.

Materials and methods

CLINICAL SAMPLES

To define the prevalence of CMV infection in patients with AIDS we prospectively collected multiple tissues from all such patients undergoing necropsies at this institution. For these clinicopathological studies, we aimed to collect up to 14 organs from each necropsy (lymph node, spleen, brain, lung, heart, kidney, adrenal, oesophagus, duodenum, colon, pancreas, liver, stomach, and salivary gland). A total of 139 organs were available from 11 unselected human immunodeficiency virus (HIV) positive patients (median, 14 organs/patient; range, 9-14). The median CD4 count at death was 10/mm³ (range, 0-20). Nine patients had been prescribed zidovudine during their illness but all died before protease inhibitor drugs became available.¹⁹

HISTOPATHOLOGICAL EXAMINATION

The tissue samples were placed into buffered formalin during the course of a standard postmortem examination. After a minimum period of 48 hours in fixative, blocks were taken and processed through to paraffin wax. Sections were cut at 5 μ m, stained with haematoxylin and eosin (Lillie's modification of Mayer's

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Table 1 The number of organs that contained CMV, HHV-6, or HHV-7 DNA related to the presence of owl's eye inclusions

Owl's eye inclusions	CMV DNA			HHV-6 DNA			HHV-7 DNA		
	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total
Yes	10	0	19	13	6	19	9	10	19
No	75	45	120	87	33	120	83	37	120
Total	94	45	139	100	39	139	92	47	139
	p = 0.0004			p = 0.78			p = 0.07		

CMV, cytomegalovirus; HHV, human herpesvirus; neg, negative; pos, positive.

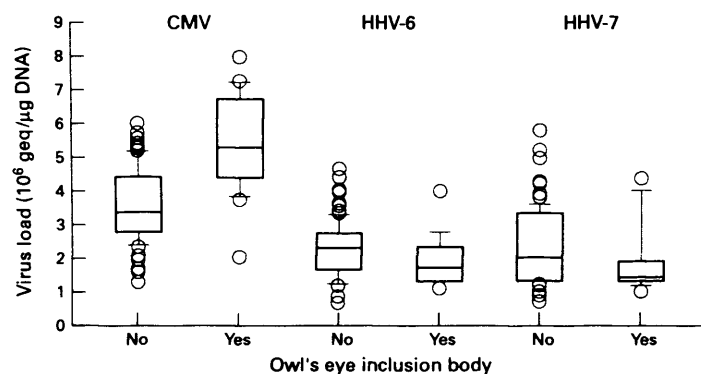


Figure 1 Box plots illustrating the relations between viral loads for three β -herpesviruses and the presence of owl's eye inclusions. The horizontal lines display the 10th, 25th, 50th (thick line), 75th, and 90th centiles; the boxes encompass 50% of the values; data points illustrate individual outlying values. CMV, cytomegalovirus; HHV, human herpesvirus.

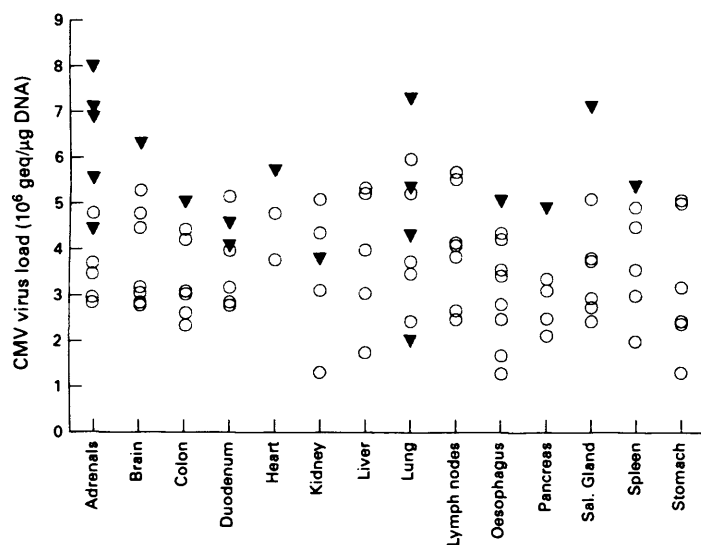


Figure 2 Association between the quantity of cytomegalovirus (CMV) DNA and the presence of owl's eye inclusions in particular organs. Closed triangle, CMV load associated with owl's eye inclusions; open circle, CMV load associated with negative staining for owl's eye inclusions.

haematoxylin), and examined. The presence of typical owl's eye inclusions was recorded as a positive finding. If the morphological features were considered inconclusive, immunohistochemical staining (Dako monoclonal antibody; Dako, Cambridge, UK) was carried out to provide confirmatory evidence. All of the sections were read by a single observer (JMCL).

EXTRACTION OF DNA

From each tissue, a block approximately $5 \times 5 \times 5$ mm was finely dissected and washed three times with sterile phosphate buffered saline. DNA was extracted from the tissue

using the Promega Wizard DNA preparation kit (Promega, Southampton, UK), according to the manufacturer's instructions. The DNA was resuspended in water and $1 \mu\text{g}$ DNA used for all subsequent analyses (equivalent to $\sim 1.5 \times 10^5$ diploid cells).

METHODS FOR PCR AND QCPCR

The methods used to detect CMV, HHV-6, and HHV-7, both qualitatively and quantitatively, have been described in detail elsewhere.¹⁵⁻¹⁷ Briefly, the PCRs amplify genes UL55, U67, and U42 of CMV, HHV-6, and HHV-7, respectively. The sensitivity of the methods was comparable, with the ultimate sensitivity of detection of CMV being 5 geq/μg DNA, whereas the HHV-6 and HHV-7 QCPCR assays were capable of detecting 2 geq/μg DNA.

STATISTICAL METHODS

Contingency tables were constructed to show the relations between visualisation of inclusion bodies and the presence of each β -herpesvirus. The significance of any observed differences was assessed by means of the χ^2 test (or Fishers exact test where appropriate).

Among those samples that contained β -herpesvirus DNA detectable by PCR, we plotted the viral load (determined by QCPCR) for each virus according to whether or not owl's eye inclusions were seen. The significance of observed differences seen was examined by the student's *t* test.

Results

Owl's eye inclusions were seen in 19 of 139 tissues (13.5%). Inclusions were seen in organs from six of 11 patients. Inclusions were found on one or more occasion in 11 of 14 organs sampled (liver, stomach, and lymph node were negative in all cases).

Table 1 shows the results of qualitative PCR testing. There was a significant association between the detection of CMV by PCR and the presence of owl's eye inclusions ($p = 0.0004$). Of note, no inclusions were seen in tissues that were PCR negative. There was no association between the detection of HHV-6 and the presence of owl's eye inclusions, which were found in 13 of 100 (13%) HHV-6 PCR positive tissues compared with six of 39 (15%) HHV-6 PCR negative samples. For HHV-7, there was a trend for inclusions to be found less frequently in tissues that were PCR positive for HHV-7 (nine of 92; 10%) compared with those that were HHV-7 PCR negative (10 of 47; 21%). This difference was of borderline significance ($p = 0.07$).

We next analysed the relation between viral load for CMV, HHV-6, and HHV-7 in different organs and the visualisation of owl's eye inclusions in histological sections from these organs (fig 1).

The CMV viral load was significantly higher ($p < 0.001$; unpaired *t* test) in samples positive for owl's eye inclusions (mean viral load, 5.35×10^6 geq/μg DNA; range, $2-7.95 \times 10^6$ geq/μg DNA), compared with samples where no

owl's eye inclusions could be seen (mean viral load, 3.55×10^6 geq/ μ g DNA; range, $1.3\text{--}5.99 \times 10^6$ geq/ μ g DNA).

In contrast, no significant relation was found between the mean viral load for HHV-6 or HHV-7 from samples positive and negative for owl's eye inclusions. The mean viral load was slightly higher for HHV-6 (2.3×10^6 geq/ μ g DNA; range, $0.7\text{--}4.6 \times 10^6$ geq/ μ g DNA) and HHV-7 (2.3×10^6 geq/ μ g DNA; range, $0.7\text{--}5.8 \times 10^6$ geq/ μ g DNA) in tissue samples negative for owl's eye inclusions, compared with samples positive for owl's eye inclusions (HHV-6 mean viral load, 1.9×10^6 geq/ μ g DNA; range, $1.1\text{--}3.9 \times 10^6$ geq/ μ g DNA; HHV-7 median viral load, 1.8×10^6 geq/ μ g DNA; range, $1\text{--}4.3 \times 10^6$ geq/ μ g DNA).

Finally, we examined in detail the quantitative relation between CMV and the presence of inclusion bodies in particular organs (fig 2). Although the numbers of individual organs were small, in general, inclusions were seen in samples with high viral loads, with the exception of lung tissues.

Discussion

The results of our investigation confirm the high specificity of owl's eye inclusions for the diagnosis of CMV organ involvement. Specifically, the presence of inclusions correlated strongly with the detection of CMV DNA by PCR and did not correlate with the detection of HHV-6 or HHV-7 DNA by PCR. We conclude that the more recently described members of the β -herpesvirinae either do not produce owl's eye inclusions that can be confused with those of CMV, or that their incidence is so low as to make them undetectable by PCR. Although these results support the continued use of inclusion body detection in clinical practice,²⁰ it should be noted that the sensitivity of detecting inclusions is relatively low in that only 19 of 94 (20%) organs that contained detectable CMV DNA also had inclusions present. This observation confirms a report from 25 years ago that cell culture is approximately six times more sensitive than histology for detecting CMV in postmortem tissues.²¹ Our QPCR studies showed that inclusions were found significantly more frequently in tissues that contained high viral loads, which presumably reflects the difficulty of finding rare virus producing cells among a large background of uninfected cells.

This work is important because it investigates the specificity of detecting owl's eye inclusions, which is part of the internationally agreed case definition of CMV disease.²⁰ Recent reports suggest that CMV disease is

more common among patients co-infected with HHV-7^{12,13} or HHV-6.¹⁴ One possible explanation for these observations could have been that HHV-6 and HHV-7 might themselves produce intranuclear inclusions and so lead to a false association with CMV disease. Our results show that this is not the case and so should facilitate future studies on the possible interactions between members of the β -herpesviruses in vivo.

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A Randomized, Controlled Trial Comparing Ganciclovir to Ganciclovir Plus Foscarnet (Each at Half Dose) for Preemptive Therapy of Cytomegalovirus Infection in Transplant Recipients

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Forty-eight patients who provided 2 consecutive blood samples that tested positive for cytomegalovirus DNA by polymerase chain reaction (PCR) were randomized to receive either full-dose ganciclovir (5 mg/kg intravenously [iv] twice daily) or half-dose ganciclovir (5 mg/kg iv once daily) plus half-dose foscarnet (90 mg/kg iv once daily) for 14 days. In the ganciclovir arm, 17 (71%) of 24 patients reached the primary end point of being CMV negative by PCR within 14 days of initiation of therapy, compared with 12 (50%) of 24 patients in the ganciclovir-plus-foscarnet arm ($P = .12$). Toxicity was greater in the combination-therapy arm. In patients who failed to reach the primary end point, baseline virus load was 0.77 log₁₀ higher, the replication rate before therapy was faster (1.5 vs. 2.7 days), and the viral decay rate was slower (2.9 vs. 1.1 days) after therapy. Bivariable logistic regression models identified baseline virus load, bone-marrow transplantation, and doubling time and half-life of decay as the major factors affecting response to therapy within 14 days. This study did not support a synergistic effect of ganciclovir plus foscarnet *in vivo*.

Cytomegalovirus (CMV) is a common infectious agent that, unless the patient is immunocompromised, rarely causes symptoms. After transplantation of bone marrow or solid organs, CMV can cause fever, pneumonitis, hepatitis, enteritis, or retinitis, collectively termed "CMV disease." Natural-history studies show that CMV viremia precedes CMV disease and that the peak virus load correlates strongly with the development of CMV disease [1, 2]. In multivariable statistical models, peak virus load explains the previously identified risk factors of donor/recipient serostatus [1–3]. Management strat-

egies for preventing CMV disease include giving antiviral prophylaxis to patients from the time of transplant onward [4] or using the results of virologic surveillance to identify asymptomatic patients with viremia and offering them antiviral therapy [5] before disease develops (preemptive therapy).

In our institution (Royal Free and University College Medical School, London, UK), bone marrow, liver, and renal transplant patients are tested twice weekly by use of polymerase chain reaction (PCR) [6]. Preemptive therapy with ganciclovir (5 mg/kg twice daily) is given intravenously (iv) for 14 days to patients with 2 consecutive positive results by PCR. Ganciclovir-induced neutropenia is treated by switching to foscarnet, a drug that is nephrotoxic and causes electrolyte imbalances [7]. Ganciclovir and foscarnet show *in vitro* synergistic activity against CMV [8], and a randomized trial of patients with AIDS showed significantly delayed progression of CMV retinitis in patients who received this combination [9]. We therefore hypothesized that a

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combination strategy consisting of half-dose ganciclovir and half-dose foscarnet (the regimen used for the maintenance phase of the trial in patients with AIDS [9]) may provide more-efficacious control of CMV viremia in the transplant setting, while reducing the risk of severe adverse effects when used for preemptive therapy. This article describes the results of a randomized, controlled trial designed to test this hypothesis and to investigate the virologic determinants of the outcome of therapy.

SUBJECTS AND METHODS

The general treatment of patients undergoing bone-marrow, liver, or renal transplantation at our institution is described in detail elsewhere [1–3]. Of note, no antiviral prophylaxis for CMV is given to recipients of solid organs, whereas all bone-marrow transplant recipients receive high-dose aciclovir, according to a previously published protocol [10].

Monitoring of CMV. Whole-blood samples were obtained twice weekly from all patients and were tested for CMV DNA by use of a PCR method described elsewhere [11]. The sensitivity of the assay is 200 genomes/mL of blood, a value that has been shown to identify patients at risk of future CMV disease [11]. All CMV-positive samples were quantified using a quantitative-competitive PCR described in detail elsewhere [12]. These quantitative values were not available to clinicians treating the patients enrolled in the present study.

Randomized trial design. Patients who provided 2 consecutive blood samples that tested positive for CMV by PCR for the first time after transplantation were invited to enter the randomized trial, which was approved by the local ethics committee (institutional review board of the Royal Free Hospital, London, UK). Patients were randomized to receive either full-dose ganciclovir (5 mg/kg twice daily) or half-dose ganciclovir (5 mg/kg once daily) plus half-dose foscarnet (90 mg/kg once daily) for 14 days. Doses were adjusted according to renal function. Patients received granulocyte colony-stimulating factor (G-CSF) at the discretion of the physician. Randomization was stratified by type of organ transplanted. The trial was administered by use of sealed envelopes containing the randomization code, which were opened after the patient had provided informed consent. Patients with impaired renal function (creatinine clearance, <30 mL/min), neutropenia ($<0.5 \times 10^9$ cells/L), or HIV infection were excluded from the study. Patients in either arm of the study who failed to clear their CMV viremia within 14 days of initiation of therapy could either be withdrawn from the study or be given a further course of therapy, at the discretion of the physician. The first patient was enrolled in December 1998, and the last was enrolled in February 2001. Patients who developed serious adverse events related to toxicity profiles of ganciclovir (neutropenia) or foscarnet (electrolyte disturbance or renal impairment) could be withdrawn from

the study, be switched to the other arm, or be allowed to continue receiving the study drug, with a reduced dose.

Statistical analysis. The primary end point was the proportion of patients who became CMV negative by PCR within 14 days of initiation of therapy. Secondary end points addressed the safety and tolerability of combination therapy and changes in CMV load during therapy. All analyses were by intention to treat. Comparison of continuous variables, such as virus load, between groups was achieved by use of the 2-sided *t* test. The Mann-Whitney *U* test was used for the comparison of viral replication rates. The rate constant κ , for viral growth or decay, before or after therapy, was computed assuming exponential growth/decay, as described elsewhere [13]. Doubling times and half-life of decay were calculated by use of the following formulae: $t_d = \ln(2)/\rho$ and $t_{1/2} = -\ln(2)/\kappa$, respectively. Simple linear least-squares fit regression models were used to analyze the relationship between viral replication kinetics and virus load. Factors associated with the primary end point were modelled with a univariable and multivariable logistic regression model using intention-to-treat design. Kaplan-Meier plot and a log-rank test were used to test the hypothesis that both therapy arms were equally effective.

Important practical difficulties in the coadministration of these 2 compounds were anticipated. Specifically, foscarnet and ganciclovir are incompatible in the same dilution fluid, and the low solubility of foscarnet increased the fluid challenge the patients were subjected to. For these reasons, it was decided that only a substantial superiority of the combination would be clinically significant. On the basis of historical data, 50% of patients given ganciclovir monotherapy were expected to become CMV negative by PCR within 14 days of initiation of therapy. A study size of 48 patients (24 in each arm) had 90% power to detect a statistically significant ($P < .05$) increase in this rate, to 90%. Data were analyzed with the computer program R [14].

RESULTS

Baseline characteristics. Details of the patients randomized to each therapy arm are given in table 1. The patients were well matched for sex, age, and CMV donor/recipient serostatus. Virus loads at day 0 (baseline virus load) were similar in both therapy arms (4.28 \log_{10} genomes/mL [ganciclovir] vs. 4.01 \log_{10} genomes/mL [ganciclovir plus foscarnet]; $P = .73$, Mann-Whitney *U* test). There was no difference between the time that the second sample was positive for CMV by PCR and the time of initiation of therapy, when patients were stratified according to therapy allocation (median, 4 days for ganciclovir and 5 days for ganciclovir plus foscarnet; $P = .24$, Mann-Whitney *U* test) or transplant type (median, 4 days for bone marrow and 5 days for solid organ; $P = .19$, Mann-Whitney *U* test).

Table 1. Characteristics of patients, by therapy assignment.

Characteristic	Therapy allocation	
	GCV	GCV plus FOS
No. of patients randomized, by type of transplantation	24	24
Bone marrow	8	9
Liver	8	9
Renal	8	6
Age, median (range), years	50 (2–68)	45 (2–56)
Sex, M:F	15:9	14:10
CMV IgG D/R status ^a (bone marrow, liver, renal), no. of patients		
D ⁺ /R ⁻	2 (1, 1, 0)	3 (0, 3, 0)
D ⁻ /R ⁺	4 (1, 2, 1)	6 (2, 1, 3)
D ⁺ /R ⁺	15 (6, 4, 5)	15 (7, 5, 3)
D ⁻ /R ⁻	1 (0, 1, 0)	0 (0)
Baseline CMV load, median (range), log ₁₀ genomes/mL	4.3 (2.7–5.9)	4.0 (3.0–6.2)

NOTE. +, Seropositive; -, seronegative; CMV, cytomegalovirus; D, donor; FOS, foscarnet; GCV, ganciclovir; R, recipient.

^a CMV IgG serostatus of donor is unknown for 2 patients (both renal transplant recipients).

Assessments of virological responses. The primary end point of being CMV negative (<200 genomes/mL) by PCR was reached by 17 (71%) of 24 patients randomized to receive ganciclovir and by 12 (50%) of 24 patients randomized to receive combination therapy ($P = .14$, χ^2 test). Among those who reached the primary end point, the median time until a blood sample was found to be negative for CMV was 6 days in the ganciclovir arm, compared with 5.5 days in the combination-therapy arm (median, 6 days vs. 11 days, when considering all patients, by use of a Kaplan-Meier approach; table 2). A Kaplan-Meier plot illustrating the proportions and times when patients became CMV negative within the first 14 days of therapy is shown in figure 1. Three patients in the ganciclovir-therapy arm and 1 patient in the combination-therapy arm had already become CMV negative by PCR at day 0. Two

patients (both in the combination-therapy arm) had no CMV-negative results by PCR within 50 days. Antiviral therapy was stopped in 1 patient (ganciclovir arm) at day 12. Statistically, for the primary end point, there was no difference between the 2 therapy arms. ($P = .19$, log-rank test). With respect to drug toxicity, 7 patients experienced toxicity, all of whom were in the combination-therapy arm (7/24 in the combination-therapy arm vs. 0/24 in the ganciclovir arm; $P = .009$, Fisher's exact test; table 2). All cases of switching and dose reduction because of toxicity occurred in the solid-organ transplant group (4 liver and 2 renal transplant recipients), whereas 1 patient in the bone-marrow transplant group was withdrawn from the study (ganciclovir-plus-foscarnet arm) at day 12 because of failure to engraft the donated marrow. In 6 patients who continued in the study, toxicity was compatible with the toxicity profile of

Table 2. No. of patients reaching the primary and secondary end points of the study, by therapy assignment.

Characteristic end points	Therapy allocation		
	GCV	GCV plus FOS	P
Primary, CMV negative by PCR within 14 days of initiation of therapy	17 (71)	12 (50)	.12 ^a
Secondary, patients stopping or reducing dose because of toxicity within 14 days of initiation of therapy	0 (0)	7 (29)	.009 ²
Time to CMV-negative result by PCR, median, days			
Patients reaching primary end point	6	5.5	
All patients, Kaplan-Meier	6	11	
Patients developing a second episode of CMV viremia after successful therapy (within 365 days)			
Patients reaching primary end point	1	4	.35 ^b
All patients	6	9	.53 ^b

NOTE. Data are no. (%) of patients, unless otherwise noted. CMV, cytomegalovirus; FOS, foscarnet; GCV, ganciclovir; PCR, polymerase chain reaction.

^a χ^2 test.

^b Fisher's exact test.

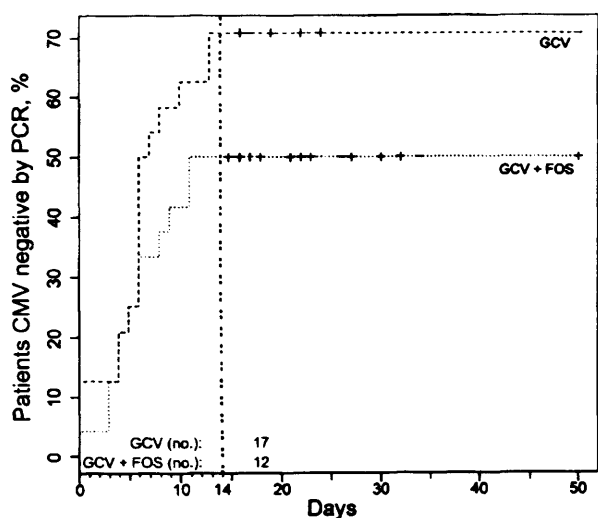


Figure 1. Kaplan-Meier analysis of time to reach the primary end point, for patients randomized to the ganciclovir (GCV) or GCV-plus-foscarnet (FOS) therapy arm. The vertical dashed line at day 14 indicates the primary end point of the study. Crosses after 14 days indicate when a patient who failed to reach the study end point became cytomegalovirus (CMV) negative.

foscarnet. Three of these patients switched to ganciclovir, and, for 3 patients, the dose was reduced (table 3).

Virologic predictors of the outcome of therapy. In 42 of the 48 patients, we were able to measure virologic parameters, such as virus load at initiation of therapy, viral growth before therapy, and viral decay rates during therapy. Patients who failed to reach the primary end point had a higher virus load at baseline (difference, 0.77 log₁₀ genomes/mL; $P = .02$, t test; figure 2). Although there was no difference in the viral replication rate (day⁻¹, ρ for ganciclovir, 0.33 vs. ρ for ganciclovir plus foscarnet, 0.43; $P = .91$, Mann-Whitney U test) or viral decay rate (day⁻¹, κ for ganciclovir, -0.42 vs. κ for ganciclovir plus foscarnet, -0.28; $P = .26$, Mann-Whitney U test) in virus load, between the 2 therapy arms, the viral decay rate was significantly higher in patients who reached the primary end

point than in those who failed to control replication to <200 genomes/mL at day 14 (day⁻¹, κ for no failure, -0.61 vs. κ for failure, -0.24, corresponding to half-life of decay of 1.1 days and 2.9 days, respectively; $P < .0002$; figure 3A). Similar results in viral decay rates were obtained when patients were analyzed according to transplant type (bone marrow vs. solid organ; data not shown). Analyses of the viral replication kinetics before therapy revealed that patients who failed to reach the primary end point had a significantly faster CMV growth rate (day⁻¹, κ for failure, 0.46 vs. κ for no failure, 0.26; $P < .002$; figure 3B). These growth rates correspond to viral doubling times of 1.51 days and 2.7 days, respectively.

The correlation between viral decay rates and baseline virus load, for patients who reached or failed to reach the primary end point, are shown in figure 4. There was a significant correlation between baseline CMV load and decay rate in each group (r^2 for failure, 0.6 vs. r^2 for no failure, 0.52; $P < .001$). In addition, when patients were stratified according to whether they had reached the primary end point, there was a strong correlation between the viral growth rate and the viral decay rate after therapy (r^2 for failure, 0.49 vs. r^2 for no failure, 0.73; $P = .001$; data not shown).

Quantifying the risks associated with poor antiviral response. Univariable linear regression models were used to identify factors associated with the primary end point (CMV negative by PCR within 14 days of initiation of therapy). In these models, higher virus load on the day of initiating antiviral therapy (odds ratio [OR], 2.39; 95% confidence interval [CI], 1.05–5.45), faster viral doubling time (t_d ; OR, 2.95; 95% CI, 1.28–6.82) before therapy, and slower half-life of decay in virus load ($t_{1/2}$; OR, 3.01; 95% CI, 1.45–6.25) after therapy were all associated with failure to reach the primary end point. Age (≤ 40 vs. > 40 years), sex, transplant type (bone marrow vs. solid organ), study drug (ganciclovir vs. combination), or delay of therapy did not appear to be associated with the primary end point (table 4). As a consequence of the strong correlation between virus load at initiation of therapy, replication rate before therapy, and viral decay rate after therapy, multivariable

Table 3. No. of patients with reported drug toxicities during the study period, clinical management parameters, and study outcome.

Transplant type	Randomized drug	Days after transplantation	Day of toxicity	Abnormal clinical parameter ^a	Attributable to study drug	Treatment	Study outcome
Bone marrow	Combination	73	12	Failure to engraft	No	Withdrawn	Failure
Liver	Combination	38	4	Creatinine, 129; magnesium, 0.38; calcium, 1.58	Yes	Switched to GCV	Failure
Liver	Combination	34	4	Creatinine, 130; magnesium, 0.51; calcium, 2.04	Yes	Switched to GCV	Failure
Liver	Combination	36	6	Creatinine, 162; magnesium, 0.52; calcium, 1.7	Yes	Switched to GCV	Failure
Liver	Combination	44	9	Creatinine, 167; magnesium, 0.57	Yes	Dose reduction	No failure
Renal	Combination	40	11	Creatinine, 154; magnesium, 0.64	Yes	Dose reduction	No failure
Renal	Combination	40	11	Creatinine, 182	Yes	Dose reduction	No failure

NOTE. GCV, ganciclovir.

^a Normal range: creatinine, 0–97 μ mol/L; calcium, 2.1–2.6 mmol/L; magnesium, 0.7–1.0 mmol/L.

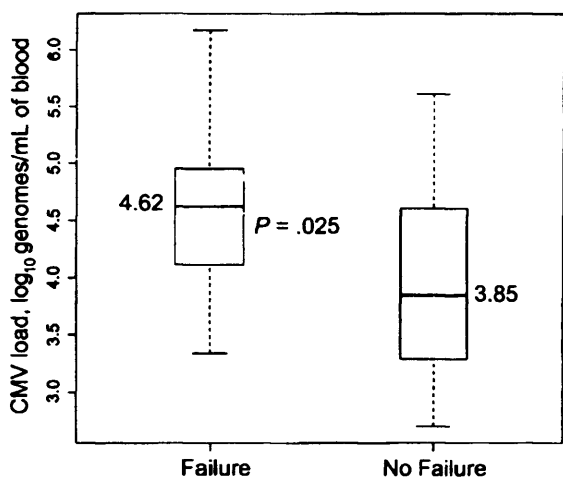


Figure 2. Box-plots illustrating the relationship between baseline virus loads for patients who reached the primary end point of becoming cytomegalovirus (CMV) negative within 14 days of initiation of therapy (no failure) and patients who failed to reach the primary end point (failure). Horizontal lines display the 10th, 25th, 50th (*thick line*), 75th, and 90th percentiles, with boxes encompassing 50% of values.

models including all these factors did not produce meaningful results. Consequently, we undertook a series of bivariable logistic regression models. The results of these models illustrate, that, in all cases, type of transplant (bone marrow) was associated with failing to become CMV DNA negative within 14 days of initiation of therapy (table 4). In addition, the measure of the viral replication (higher baseline virus load, faster doubling time, or slower viral half-life of decay) was independently associated with failure to reach the primary end point.

Subsequent episodes of CMV viremia. In 15 (31%) of 24 patients, we observed a second episode of CMV viremia within 1 year after they finished the first course of therapy. When analysis was performed according to the initial randomized drug, there was no significant difference in the number of patients who experienced a second episode of CMV viremia (ganciclovir = 6 and ganciclovir plus foscarnet = 9; $P = .5$, Fisher's exact test; table 2). However, patients who failed to reach the primary end point of the study were more likely to have a second episode of CMV viremia ($P = .01$, Fisher's exact test). In addition, among patients with a second episode of viremia, there was a trend for higher baseline virus load and faster replication rate before therapy (data not shown).

DISCUSSION

The main conclusion of this randomized, controlled clinical trial is that, for preemptive therapy in transplant recipients, the combination of half-dose ganciclovir and foscarnet therapy is not superior to ganciclovir monotherapy. The viral decay rate

after therapy was very similar between patients randomized to receive ganciclovir and those randomized to receive the combination therapy. However, with respect to the primary end point of being CMV negative by PCR by day 14, there was a trend in favor of the ganciclovir arm. Thus, the lack of a significant benefit of the combination of ganciclovir and foscarnet

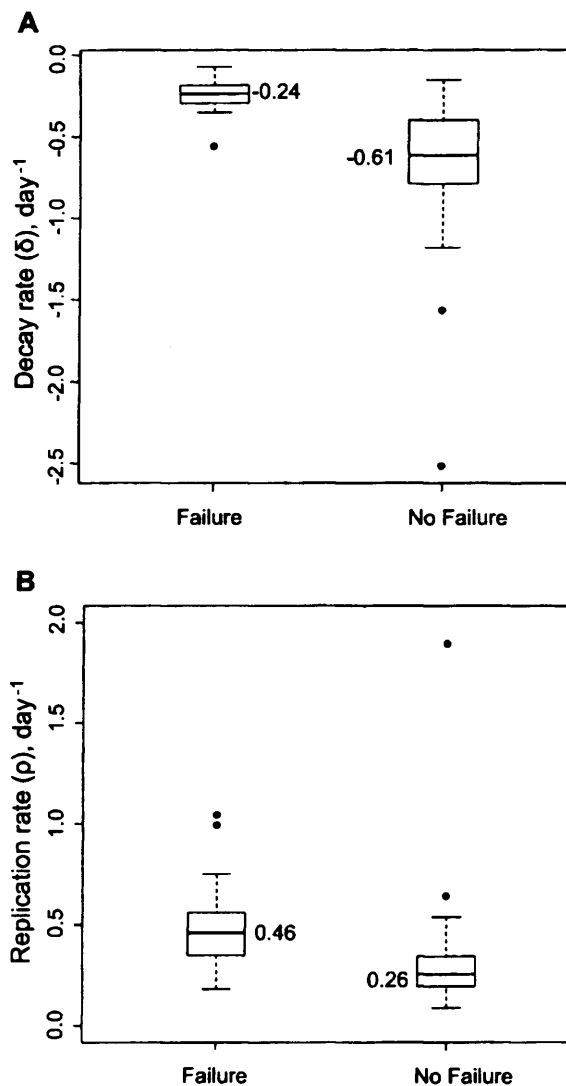


Figure 3. A and B, Box-plots showing the viral decay rate (δ) after initiating antiviral therapy (A) and the replication rate (ρ) before initiating antiviral therapy (B), for patients who reached the primary end point of becoming cytomegalovirus (CMV) negative within 14 days of initiation of therapy (no failure) and patients who failed to reach the primary end point (failure). Replication rate and decay rate were each statistically significantly different in patients who failed to reach the primary end point, compared with patients who did not fail to reach the primary end point. Horizontal lines display the 10th, 25th, 50th (*thick line*), 75th, and 90th percentiles, with boxes encompassing 50% of values. Dots represent values outside the 90% limits.

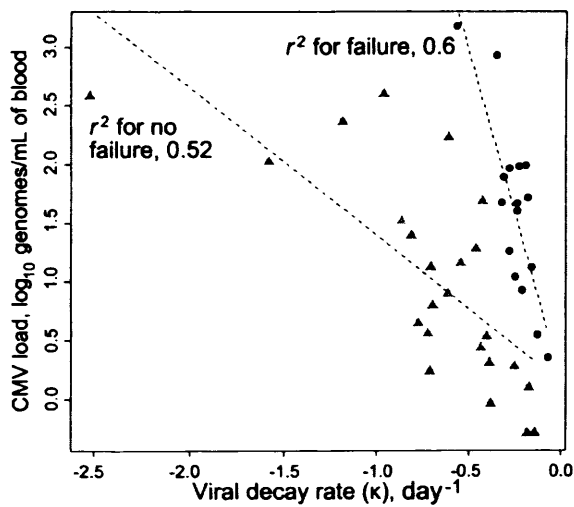


Figure 4. Scatter-diagram showing the correlation between viral decay rate (κ) and baseline cytomegalovirus (CMV) load. Data are stratified according to the study end point. A correlation line was fitted for each strata (●, failure; ▲, no failure), and the goodness of fit is expressed as an r^2 value.

could not be explained by inadequate study power. Since patients were well matched at baseline for all known prognostic variables, including donor/recipient serostatus and baseline virus load, this outcome was surprising. The hypothesis that the combination therapy might be superior to ganciclovir monotherapy was based on 2 previously published observations: (1) ganciclovir plus foscarnet is synergistic in vitro [8] and (2) the combination (using the same doses as used here) proved to be superior in controlling CMV retinitis in patients with AIDS [9]. However, we now know that extrapolation of data from in vitro studies with anti-CMV compounds can be misleading, since aciclovir, which has a poor efficacy in vitro, has been shown to be efficacious in vivo within a series of randomized, controlled clinical trials [15, 16]. Furthermore, the results of the randomized, controlled trial of ganciclovir and foscarnet in patients with AIDS retinitis, which showed a clear superiority of the combination therapy, were obtained in a group of patients who had experienced high-level CMV replication and had been pretreated with ganciclovir. We have previously shown that the rapid dynamics of CMV, coupled with prolonged persistence of low levels of ganciclovir, can result in a rapid flux of wild-type and drug-resistant virus populations and that in vitro culture of viruses in the absence of ganciclovir can result in an underappreciation of the quantity of resistant viruses present in the clinical inoculum [13, 17]. Thus, a high incidence of unrecognized resistance to ganciclovir could explain the observed differences between the trials, with the ganciclovir-plus-foscarnet arm showing improved virologic and clinical benefit in patients with AIDS retinitis, because of the ability to control

low-level ganciclovir-resistant strains of viruses with mutations in the UL97 gene. Alternatively, foscarnet may have had a modest effect on HIV replication; therefore, in retrospect, the AIDS trial may represent an early example of combination antiretroviral therapy, rather than synergistic anti-CMV activity of both drugs.

In the present study, the availability of data on virus loads enabled us to investigate virological parameters associated with therapy failure. Patients who failed to control replication to low levels (<200 genomes/mL) within 14 days of initiation of therapy had a significantly higher baseline virus load, a much faster viral growth rate before therapy (average $t_{1/2}$, 1.5 days), and a much slower viral decay rate after therapy (average $t_{1/2}$, 2.9 days), compared with patients who reached the primary end point. These observations on the importance of viral growth rate and decay after therapy, in patients with poor response to antiviral therapy, have important practical implications, and further studies are required to determine the role of host parameters in facilitating the removal of CMV-infected cells after initiating therapy.

It is known that a subset of patients treated via preemptive therapy will experience a recurrence of viremia. In the present study, this constituted 31% of the cohort. Patients who failed to reach the primary end point of the study were more likely to have a second episode of CMV viremia within the first year

Table 4. Univariable and multivariable analysis of risk factors associated with the primary end point of becoming cytomegalovirus (CMV) DNA negative by polymerase chain reaction by day 14.

Model, risk factors	OR (95% CI)	P
Univariable models		
Transplant group, bone marrow vs. solid organ	2.36 (0.70–7.94)	.17
CMV load ^a , per log ₁₀ increase	2.39 (1.05–5.44)	.038
Age, ≤40 vs. >40	1.19 (0.37–3.86)	.77
Study drug, GCV vs. GCV plus FOS	0.41 (0.13–1.35)	.144
Sex, male vs. female	0.59 (0.18–1.91)	.37
Doubling time, per day decrease	2.95 (1.28–6.82)	.01
Half-life of decay, per day increase	3.01 (1.45–6.25)	.003
Multivariable model 1		
Transplant group, bone marrow vs. solid organ	3.94 (0.92–16.92)	.064
CMV load ^a , per log ₁₀ increase	2.96 (1.01–6.15)	.048
Multivariable model 2		
Transplant group, bone marrow vs. solid organ	3.79 (0.78–18.24)	.09
Doubling time, per day decrease	3.02 (1.28–7.14)	.018
Multivariable model 3		
Transplant group, bone marrow vs. solid organ	7.46 (1.19–46.71)	.031
Half-life of decay, per day increase	3.7 (1.55–8.84)	.003

NOTE. CI, confidence interval; FOS, foscarnet; GCV, ganciclovir; OR, odds ratio.

^a Virus load measured at day of initiation of therapy.

($P = .013$, Fisher's exact test; data not shown). In addition, patients who had a second episode of viremia had a higher virus load at baseline, a higher replication rate before antiviral therapy, and a slower viral decay rate after initiating antiviral therapy (data not shown). This latter observation is similar to those of Humar et al. [18], who showed that solid-organ transplant recipients who had second episodes of viremia had a $t_{1/2}$ of 8.8 days after iv ganciclovir therapy, compared with a $t_{1/2}$ of 3.17 days for patients with a single episode of CMV viremia.

In conclusion, this trial has shown that, at the doses used, combination antiviral therapy with ganciclovir plus foscarnet for CMV viremia does not appear to control viral replication better than does ganciclovir monotherapy. Other investigators have reported uncontrolled studies of full-dose therapy with ganciclovir plus foscarnet [19] or increasing doses of foscarnet plus constant ganciclovir, at the doses used here [20]. Future randomized trials could consider using 1 of these regimens as a way of obtaining better control of CMV replication after transplantation.

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Kinetics of Cytomegalovirus Load Decrease in Solid-Organ Transplant Recipients after Preemptive Therapy with Valganciclovir

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The availability of valganciclovir (VGCV) has significantly simplified the treatment of human cytomegalovirus (HCMV) infection after solid-organ transplantation. We show that there was no difference in the kinetics of the decrease in HCMV load after preemptive therapy with VGCV in 22 solid-organ transplant recipients ($T_{1/2} = 2.16$ days), compared with that in 23 patients treated with intravenous ganciclovir (GCV) ($T_{1/2} = 1.73$ days; $P = .63$). Preemptive therapy with VGCV provides control of HCMV replication that is comparable to that achieved with preemptive intravenous therapy with GCV.

Human cytomegalovirus (HCMV) remains an important cause of morbidity after solid-organ transplantation. Infection has been associated with a number of direct and indirect effects in immunocompromised hosts, including hepatitis, prolonged pyrexia, and acute and chronic graft rejection [1]. HCMV replicates rapidly in the human host, and the viral load is directly related to the probability of disease development [2–4]. Ganciclovir (GCV) has become one of the most commonly used antivirals to control HCMV replication in solid-organ transplant recipients. Therapeutic approaches include prophylaxis, which is usually administered to patients at high risk of developing HCMV disease, and preemptive therapy, in which the drug is administered on the basis of the detection of active

HCMV replication by sensitive laboratory methods such as the antigenemia assay or polymerase chain reaction (PCR). Recently, controlled clinical trials of the valine ester prodrug of GCV, valganciclovir (VGCV), have shown that it is effective in the treatment of HCMV in HIV-infected patients [5, 6] and in the prophylaxis of high-risk solid-organ transplant recipients [7]. VGCV offers many advantages over existing formulations of GCV—high plasma levels of drug can be achieved through oral dosing, and patients no longer require extensive hospitalization for intravenous (iv) infusions. A dose of 900 mg of VGCV provides plasma GCV exposure that is approximately comparable to a 5-mg/kg dose of iv GCV [8, 9]. At present, there have been no trials that have compared preemptive therapy with VGCV with that of iv GCV in solid-organ transplant recipients. However, increasingly, VGCV is being used in such a treatment modality. We therefore investigated whether the kinetics of the control of HCMV replication in patients receiving VGCV were comparable to those that we have previously observed in patients receiving iv GCV [10].

Subjects, materials, and methods. In a retrospective non-randomized study, renal- or liver-transplant recipients receiving either oral VGCV (900 mg twice/day) or iv GCV (5 mg/kg twice/day) for the treatment of HCMV infection were identified between October 2001 and September 2003. Patients were monitored for HCMV DNAemia at least twice weekly, when they were hospitalized, or when they attended the outpatient clinic. HCMV infection was defined as 2 consecutive positive HCMV PCR results (cutoff, 200 genomes/mL). HCMV PCR was done on a routine basis with an in-house TaqMan (ABI)-based method adapted from our previously published method [11]. Briefly, DNA was extracted from 200 μ L of whole blood by use of a Qiagen extraction kit (Minden), according to the manufacturer's instructions. Real time-amplification of HCMV DNA used glycoprotein B-specific primers, as described elsewhere [12] (5'-GAGGACAACGAAATCCTGTTGGGCA-3' [gB1] and 5'-TCGACGGTGGAGATACTGCTGAGG-3' [gB2]). The 150-bp product was detected in real time by use of a 29-mer TaqMan probe (5'-CAATCATGCGTTTGAAGAGGTAGTCCACG-3' [gb-P3]), which was labeled at the 5' end with 6-FAM and at the 3' end with TAMRA. The conditions for the PCR were as follows: 2.5 μ L of 1 \times PCR buffer (that contained 1.5 mmol/L MgCl₂; Qiagen), 5 μ L of MgCl₂ (25 mmol/L), 7.5 μ L of dNTPs (6.25 mmol/L each nucleotide), 1 μ L of gB1 and gB2 (15 pmol/ μ L), 1 μ L of gb-P3 (5 pmol/ μ L), and 0.25 μ L of HotStarTaq polymerase (0.25 IU/ μ L; ABI) made up to a final volume of 20 μ L with sterile water. Then, 5 μ L of extracted or control

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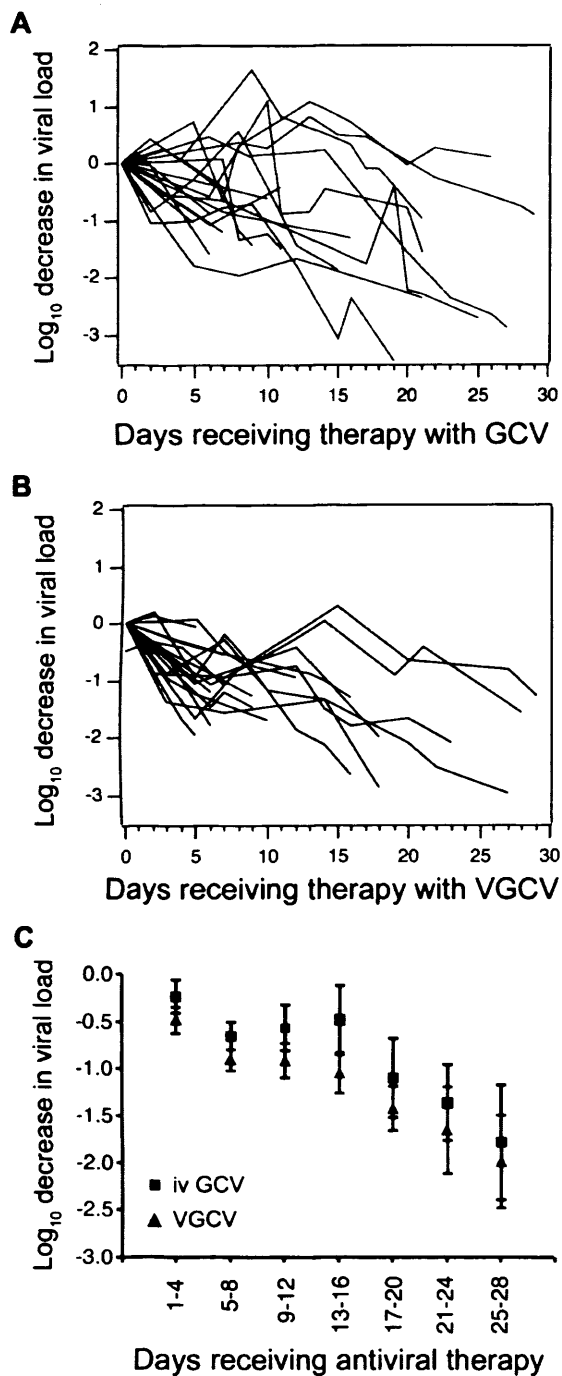


Figure 1. A, Decrease in human cytomegalovirus (HCMV) load from baseline, after the initiation of antiviral therapy for individual patients receiving intravenous (iv) ganciclovir (GCV; A) or oral valganciclovir (VGCV; B). The change in HCMV load from baseline is plotted against days receiving antiviral therapy (data shown for the first 28 days of antiviral therapy). C, Log₁₀ decrease in HCMV load after preemptive therapy with VGCV (900 mg twice daily) or iv GCV (5 mg/kg twice daily). The mean reduction in HCMV load (± 1 SD) is shown for each 3-day window after therapy, for the 28 days of therapy. Squares, iv GCV; triangles, VGCV.

DNA was added to each reaction before the PCR. In addition each TaqMan PCR run contained a dilution series of cloned HCMV gB DNA in triplicate of $1-10^4$ genomes [13]. PCR cycling conditions were 2 min at 50°C, 10 min at 95°C, and 60 cycles of 15 s at 95°C and 15 s at 60°C. All clinical samples were analyzed in duplicate, and the average HCMV load was calculated by use of the sequence detection system software available on the ABI 7700 platform.

Anti-HCMV therapy was recommended after 2 consecutive positive HCMV PCR results were obtained and was continued until 2 consecutive negative HCMV PCR results were obtained. The choice of anti-HCMV drug was made by the treating physician. The dose of VGCV and GCV was adjusted according to the renal function of the patient, as recommended by the manufacturer.

The length of a viremic episode was defined as the interval between the last negative HCMV PCR result, followed by at least 2 consecutive positive results until the first negative PCR result. Treatment delay was defined as the time from the first positive PCR result until the initiation of antiviral therapy. Baseline HCMV load was defined as the HCMV load measured at the time of starting antiviral therapy. A linear curve was fitted through all available HCMV load data before and after antiviral therapy. The doubling time or half-life was calculated by use of standard exponential growth or decay functions [4]. Groups with continuous variables were compared by use of the Mann-Whitney *U* test. Difference in proportions between groups were calculated by use of Fisher's exact test. All statistical analysis was performed as intent-to-treat analysis with the software R [14].

Results. During the period from September 2001 to October 2003, 22 patients (15 liver- and 7 renal-transplant recipients) received preemptive therapy with VGCV (900 mg twice daily). During the same time period, a further 23 patients (12 liver- and 11 renal-transplant recipients) received preemptive therapy with iv GCV (5 mg/kg twice daily). In all patients studied, HCMV loads were determined twice per week before and during therapy. With the exception of 2 patients (both of whom received iv GCV), patients received antiviral therapy until they became negative for HCMV by PCR. A total of 107 samples were quantified before the initiation of antiviral therapy (median, 3 samples/patient), and a further 177 HCMV PCR-positive samples were quantified after the start of antiviral therapy (median, 4 samples/patient).

The demographic characteristics of the 2 treatment groups were well matched in terms of treatment allocation (23 GCV vs. 22 VGCV recipients), age (mean, 43.8 years for GCV recipients vs. 49.3 years for VGCV recipients), and sex (GCV recipients, 14 men/9 women; VGCV recipients, 9 men/13 women). Immunosuppressive regimens were equally distributed within each treatment group ($P = .57$), with patients administered a reg-

Table 1. Virological response after the initiation of antiviral therapy, according to treatment.

Characteristic	Treatment		<i>P</i> ^a
	iv GCV	VGCV	
Time to negative PCR, days	14 (2–230)	15 (2–110)	.86
Treatment length, days	16 (1–33)	20 (6–49)	.19
Half-life, days	1.73 (0.87–11.55)	2.16 (0.75–6.93)	.63
Log ₁₀ decrease, genomes/mL			
Day 7	–0.64 (–1.79 to 0.57)	–1.07 (–1.96 to –0.07)	.14
Day 14	–1.17 (–3.07 to 1.07)	–0.98 (–2.12 to 0.29)	.92

NOTE. Data are expressed as median (range). GCV, ganciclovir; PCR, polymerase chain reaction; VGCV, valganciclovir.

^a Mann-Whitney *U* test; significance was set at *P* ≤ .05.

imen that contained either cyclosporin (GCV recipients, *n* = 5; VGCV recipients, *n* = 3) or tacrolimus (GCV recipients, *n* = 16; VGCV recipients, *n* = 17). More patients with primary HCMV infections (donor HCMV positive [D⁺], recipient HCMV negative [R⁻]) received GCV (*n* = 7) than VGCV (*n* = 2); however, this difference did not reach statistical significance (*P* = .27, Fisher's exact test). Treatment groups were well matched for D⁻R⁺ (4 GCV recipients and 4 VGCV recipients) and D⁺R⁺ (12 GCV recipients and 14 VGCV recipients) combinations. Virological parameters before starting antiviral therapy were comparable for HCMV load at treatment initiation (GCV, 3.55 log₁₀ genomes/mL vs. VGCV, 3.81 log₁₀ genomes/mL; *P* = .67), peak HCMV load (GCV, 3.87 log₁₀ genomes/mL vs. VGCV, 4.15 log₁₀ genomes/mL; *P* = .68), and doubling time (GCV, 2.03 days vs. VGCV, 1.82 days; *P* = .47). The treatment delay was 8 days in the GCV group and 9 days in the VGCV group (*P* = .98).

After therapy, patients who had received VGCV had a median half-life of decrease in HCMV load of 2.16 days, compared with a median half-life of decrease in HCMV load of 1.73 days in patients who received iv GCV (*P* = .63). The median decrease in HCMV load at day 7 was –1.07 log₁₀ genomes/mL in patients who received VGCV, compared with –0.65 log₁₀ genomes/mL in patients who received iv GCV, although this difference did not reach statistical significance (*P* = .14). At day 14, the decrease in HCMV load was similar in both treatment groups (GCV, –1.17 log₁₀ genomes/mL vs. VGCV, –0.98 log₁₀ genomes/mL; *P* = .92). Overall, the time to become PCR negative for HCMV was comparable between the 2 groups (median, 14 days for GCV vs. 15.5 days for VGCV; *P* = .86).

Individual decrease profiles for patients receiving preemptive therapy with iv GCV or VGCV are shown in figure 1A, and a composite figure summarizing all patients is shown in figure 1B. As noted in table 1, the decrease rates of HCMV load between the 2 treatment groups were almost identical.

Discussion. In the absence of data from randomized, controlled, clinical trials of VGCV, we used a single center's ex-

perience of preemptive therapy to compare the posttherapy kinetics of HCMV replication in solid-organ transplant recipients receiving either VGCV or iv GCV. We reasoned that, because VGCV use was becoming more widespread in the preemptive-therapy setting, it was important to show that the ability to control HCMV replication rapidly and effectively with VGCV was comparable to that achieved with iv GCV. The results clearly show that VGCV (900 mg twice daily) and iv GCV (5 mg/kg twice daily) have similar efficacy levels for the control of HCMV replication in this therapeutic setting. These results concur with the pharmacokinetics of VGCV observed in liver-transplant recipients and in HIV-infected patients—that is, a 900-mg dose of VGCV produces plasma GCV levels comparable to those produced by a 5-mg/kg iv dose of GCV. We have previously shown that replication events occurring before the initiation of preemptive therapy can have a substantial effect on the observed response to therapy [15]. Hence, rapid viral replication (a fast doubling time) and high HCMV load at the start of therapy are both associated with a slower rate of decrease after the initiation of therapy. Such events are consistent with the non-steady-state viral dynamics that are apparent during the period when HCMV loads are 200–10,000 genomes/mL and are reflected in the overshoot in HCMV load frequently observed after the initiation of therapy in the D⁺R⁻ setting. In the present analyses, HCMV load at the initiation of therapy and the average doubling time of virus before therapy were well matched between patients who received VGCV and those who received iv GCV. The present study therefore provides the first detailed assessment of HCMV kinetics after preemptive therapy with VGCV in solid-organ transplant recipients and emphasizes the rapid control of HCMV replication that is achievable. However, for completeness, more examples of the control of replication by VGCV in the D⁺R⁻ setting is warranted. However, there was no difference in the recurrence of viremia between patients who received GCV and those who received VGCV (7/23 vs. 5/22). The inclusion of measures of viral dynamics in future clinical trials of VGCV in other clinical

settings will facilitate our understanding of the factors required for the successful control of HCMV in transplant recipients.

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