การจำแนกไทป์ของไซโตเมกาโลไวรัสจากยีนไกลโคโปรตีนบี และการกลายพันธุ์ของยีนยูแอล97ในตัวอย่างสิ่งส่งตรวจที่โรงพยาบาลจุฬาลงกรณ์

นางสาวสุวิมล   จันทรอาภรณ์กุล

TYPING OF HUMAN CYTOMEGALOVIRUS gB GENOTYPES (gB) AND UL97 MUTATION FROM CLINICAL SPECIMENS AT KING CHULALONGKORN MEMORIAL HOSPITAL

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Medical Microbiology

(Inter-Department)

Graduate School

Chulalongkorn University

Academic Year 2004

This Thesis	**Typing of Human Cytomegalovirus gB genotypes (gB) and UL97 Mutation from Clinical Specimens at King Chulalongkorn Memorial Hospital**
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สุวิมล จันทรอาภรณ์กุล : การจำแนกไทป์ของไซโตเมกาโลไวรัสจากยีนไกลโคโปรตีนบี และการกลายพันธุ์ของยีนยูแอล 97ในตัวอย่างสิ่งส่งตรวจที่โรงพยาบาลจุฬาลงกรณ์ (TYPING OF HUMAN CYTOMEGALOVIRUS gB GENOTYPES (gB) AND UL97 MUTATION FROM CLINICAL SPECIMENS AT KING CHULALONGKORN MEMORIAL HOSPITAL) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. ภาวพันธ์ ภัทรโกศล; 123 หน้า. ISBN 974-17-6506-1

การติดเชื้อ Human cytomegalovirus (HCMV) เป็นสาเหตุสำคัญในการเกิดโรค และการเสียชีวิตในกลุ่มผู้ป่วยที่มีระบบภูมิคุ้มกันต่ำ ถึงแม้ว่า HCMV มีเพียง 1 ซีโรไทป์ แต่พบว่ามีความหลากหลายของสารพันธุกรรมในระหว่างสายพันธุ์ การแบ่งชนิดของ HCMV อาศัยความแตกต่างทางด้านสารพันธุกรรมในส่วนของยีนที่ควบคุมการแสดงออกของ envelope glycoprotein โดยเฉพาะในส่วนของ Glycoprotein B (gB) ซึ่งสามารถแบ่งได้ 4 ชนิด Ganciclovir (GCV) เป็นยาที่นิยมใช้ในการรักษา และป้องกันโรคที่เกิดจากการติดเชื้อ HCMV การกลายพันธุ์ของหมู่อะมิโนของ UL97 phosphotransferase มีผลทำให้ไวรัสดื้อต่อยา GCV ในประเทศไทยยังไม่มีข้อมูลการจำแนกชนิด และการกลายพันธุ์ของ UL97 ของ HCMV มาก่อน จึงเป็นที่น่าสนใจที่จะทำการศึกษา

การจำแนกไทป์ HCMV ด้วยวิธี nested PCR-RFLP จากตัวอย่างสิ่งส่งตรวจทั้งหมด 161 ตัวอย่าง สามารถจำแนกได้ 113 (70.19 %) ตัวอย่างจากผู้ป่วย 96 ราย พบการติดเชื้อไทป์ผสม (mixed genotypes) สูงที่สุด (39/113, 34.51%) รองลงมาคือ gB1 (37/113, 32.74 %) gB3 (17/113, 15.04 %) gB2 (12/113, 10.62) และ untype หรือเรียกว่า UT (8/113, 7.08 %) ตามลำดับ ไม่พบการติดเชื้อของ gB4 ถึงแม้ว่าพยายามแยก gB4 จากไทป์ผสมด้วยวิธีโคลนนิ่ง (cloning) การศึกษานี้พบรูปแบบการตัดเอนไซม์จำเพาะแบบใหม่ 2 รูปแบบ (UT1 และ UT2) โดยเฉพาะ UT1 ยังไม่มีรายงานมาก่อน เมื่อนำลำดับเบส (sequence) ของ UT1 และ UT2 มาทำ Phylogenetic tree พบว่าลำดับเบสของทั้ง 2 ชนิดจัดอยู่ในกลุ่มเดียวกับ gB1 มีความเหมือนประมาณ 97 % นอกจากนี้สายพันธุ์ที่อยู่ในไทป์เดียวกันมีลำดับเบสเหมือนกันมากกว่า 97 % และเหมือนกันประมาณ 71 %-75 % ในไทป์ที่ต่างกัน ในการศึกษาลำดับเบสพบว่ามี 5 สายพันธุ์ที่มีผลการจำแนกไทป์แตกต่างจากวิธี RFLP คือพบว่ามีลำดับเบสที่ใกล้เคียงกับ gB3 ใน 1 ตัวอย่างที่เป็น gB1 และ 4 ตัวอย่างที่เป็น gB2 ความหลากหลายภายในไทป์ (Intragenotypic variation) นอกจากพบในระหว่างตัวอย่างสายพันธุ์ (clinical strain) แล้วยังสามารถพบได้ภายในสายพันธุ์ที่มาจากคนเดียวกันโดยวิธีโคลนนิ่ง

การศึกษาการกลายพันธุ์ของ UL97 โดยวิธี sequencing พบว่ามีการแทนที่ (substitution) ของลำดับอะมิโนทั้งหมด 10 ตำแหน่ง โดย 8 ตำแหน่งเป็นตำแหน่งที่พบใหม่ อีกสองตำแหน่งคือ 590 และ 601 เป็นตำแหน่งที่มีความเกี่ยวข้องกับการดื้อยา GCV ของไวรัสซึ่งจะมีผลเมื่อ Ala ถูกแทนที่ด้วย Thr ที่ตำแหน่ง 590 และ Thr ถูกแทนที่ด้วย Met ที่ตำแหน่ง 601 แต่ในที่นี้ถูกแทนที่ด้วย Glu และ Lys ตามลำดับ อย่างไรก็ตามในการศึกษานี้ไม่สามารถระบุได้ว่าการแทนที่ของลำดับอะมิโนทั้ง 10 ตำแหน่งมีผลต่อการดื้อยา GCV ของไวรัส

โดยสรุปการศึกษาครั้งนี้พบว่ามีการติดเชื้อ HCMV แบบผสมเป็นส่วนใหญ่ซึ่งอาจเกิดจากการกลายพันธุ์ของ HCMV ในผู้ป่วย หรือเกิดจากการติดเชื้อซ้ำของไวรัสสายพันธุ์ใหม่ การจำแนกไทป์ด้วยวิธีศึกษาลำดับเบส (sequencing) ยังคงเป็นวิธีที่ดีที่สุด และนอกจากการหาลำดับเบสแล้วการศึกษาการกลายพันธุ์ของ UL97 ที่มีผลต่อการดื้อยา GCV ของไวรัสจำเป็นต้องมีผลของการแสดงออกของการดื้อยา (drug resistant phenotypic effect) ร่วมด้วย

สหสาขาวิชา..สหสาขาวิชาจุลชีววิทยาทางการแพทย์...**ลายมือชื่อนิสิต**…………………………………………….

สาขาวิชา……จุลชีววิทยา…………………………………..**ลายมือชื่ออาจารย์ที่ปรึกษา**………………………………..

ปีการศึกษา…….2547………………………………

SUWIMON CHANTARAARPHONKUN: THESIS TITLE: TYPING OF HUMAN CYTOMEGALOVIRUS gB GENOTYPES (gB) AND UL97 MUTATION FROM CLINICAL SPECIMENS AT KING CHULALONGKORN MEMORIAL HOSPITAL. THESIS ADVISOR: ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D.   123 pp. ISBN 974-17-6506-1

Human cytomegalovirus (HCMV) infection is a major cause of morbidity and mortality in immunocompromised patients. Although HCMV is believed to have only one serotype, genomic variants among clinical strains occurred throughout entire genome. Recently, sequence polymorphism of its envelope glycoproteins especially gB as commonly used to do genotyping of HCMV. There were at least 4 different gB genotypes. For treatment of HCMV associated diseases and preemptive treatment of infection, ganciclovir (GCV) is the most widely used. Acquisition of mutation in HCMV UL97 phosphotransferase appears to be a crucial step in the selection of GCV resistant strain. In Thailand, HCMV genotyping and HCMV UL97 mutation have never been studied before, therefore these studies are interesting.

HCMV gB genotyping by nested PCR-RFLP was successfully done in 113 of 161 specimens (113/161, 70.19 %) from 96 patients. The infection of mixed genotypes was the most prevalent (39/113, 34.51 %), followed by gB1 (37/113, 32.74 %), gB3 (17/113, 15.04 %), gB2 (12/113, 10.62 %), and untype, designated as UT (8/113, 7.08 %). None of gB4 has been detected, although we attempted to find gB4 from mixed genotypes by cloning method. In addition, 2 new gB restriction patterns (UT1 and UT2) were identified. Both of them were clustered nearly branch of the same origin to gB1 with approximately 97 % homology. Accordingly, sequences among clinical strains within each group had more similarly than 97 % and about 71 %-75 % between strains of the differing group. Based on sequencing, 5 clinical strains were showed distinct gB genotype determining by RFLP, 1 gB1 and 4 gB2 sequences were gB3 instead. Intragenotypic variation of HCMV gB genotype was shown among clinical samples as well as within the same clinical sample by using cloning method.

Study UL97 mutation related to GCV resistance by sequencing demonstrated 8 out of 10 amino acids substitutions were novel positions. Only 2 positions have been reported to associate with HCMV GCV resistance, Ala substitution by Thr (Ala 590 Thr) and Met substitution by Thr (Thr 601 Met). In our study, they were substituted with Glu (Ala 590 Glu) and Lys (Thr 601 Lys). Therefore, all of 10 UL97 mutations in this present work were not known whether those would confer to GCV resistance.

In conclusion, mixed HCMV genotypes were found predominantly in this study, suggesting the mutation of HCMV within a patient or the possibility of reinfection of new type / strain. Moreover, sequencing method is still the best method for HCMV gB genotyping. Besides sequencing, the study of UL97 mutation conferring GCV resistance needs the drug resistant phenotypic effect.

Inter-Department…Medical..Microbiology…**Student's signature**……………………………………

Field of study…..……Medical..Microbiology…**Advisor's signature**……………………………………

Academic year…......2004……………………

ACKNOWLEDGEMENTS

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ABBREVIATIONS

aa	Amino acid
AIDS	Acquired immunodeficiency syndrome
BAL	Broncoalveolar lavage
BMT	Bone marrow transplant
bp	Base pair
°C	Degree Celsius
Ca	Calcium
CaCl$_2$	Calcium chloride
CD	Cluster of differentiation
CDV	Cidofovir
CSF	Cerebrospinal fluid
CTLs	Cytotoxic T lymphocytes
DB	Dense bodies
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DW	Distilled water
E (β)	Early
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
et al	et alii
ETT	Endotracheal tube
fg	Framtogram
FFWO	Fusion-form-without assay
gB	Glycoprotein B
GBS	Guillain-Barre Syndrome
gc	Glycoprotein complex

ABBREVIATIONS (continued)

GCV	Ganciclovir
gH	Glycoprotein H
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O
GVHD	Graft versus host disease
HCMV	Human cytomegalovirus
HF	Human fibroblast
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HSPGs	Heparan sulfate proteoglycans
HSV	Herpes simplex virus
IE (α)	Immediate early
IFN-γ	Interferon gamma
IL-2	Interleukin 2
ISG	Interferon stimulating gene
Kbp	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodalton
L (γ)	Late
LB	Luria-Bertani
M	Molar
MCP	Major capsid protein
MCMV	Murine cytomegalovirus
MDMs	Monocyte-derived macrophages
MgCl$_2$	Magnesium chloride
MHC	Major histocompatibility complex
μg	Microgram
mg	Milligram
μl	Microlitre

ABBREVIATIONS (continued)

ml	Millilitre
mm	Millimetre
mM	Millimolar
MUT	Mixed unclassified types
NAb	Neutralizing antibody
ng	Nanogram
nt	Nucleotide
ng	Nanogram
nm	Nanometre
OD	Optical density
ORFs	Open reading frames
pAP	Assembly protein precursor
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PFA	Foscanet
pg	Picogram
pmole	Picomole
pp	Phosphoprotein
RFLP	Restriction fragment length polymorphism
rpm	Round per minute
TBE	Tris-borate ethylenediamine tetraacetic acid
Tris	Tris-(hydroxymethyl)-aminoethane
UL	Unique long
US	Unique short
UT	Untype
vol	volume
w	with
WBC	White blood cell
w/o	without

CHAPTER I

INTRODUCTION

Human cytomegalovirus (HCMV) belongs to the beta subfamily of herpesviridae. It has strict species specificity. Its nucleocapsid containing a linear double-stranded DNA of 230 kilobase pairs is surrounded by a proteinaceous tegument or matrix. Those components are enclosed in a lipid bilayer envelope carrying a number of virus encoded glycoproteins such as gB, gH, gL etc. (1).

Clinical manifestations can be seen following primary infection, reinfection or reactivation. The virus enters via the epithelium of the upper alimentary, respiratory or genitourinary tracts. Hematogeneous spreading is typically followed by infection of ductal epithelial cells such as salivary glands, kidney tubules, cervix, testes or epididymis (2). Infections are usually kept under control by the immune system. However, total HCMV clearance is rarely performed, and the viral genome remains at selected site in a latent state. During HCMV latency, viral genome can be detected but reproductive virus can not be determined (3). Recurrent infection might occur when immune system decreases, as a cause of chronic persistent infection (4). During primary, recurrent or persistent infection, HCMV is shed in multiple body fluids, such as saliva, urine, tears, cervicovaginal fluid, breast milk and semen (2, 5). Natural transmission occurs by direct or indirect person to person contact, such as intrauterine transmission, blood transfusion, sexual transmission, etc.

HCMV is a widespread pathogen responsible for HCMV infection between 50-100% of normal population worldwide (6). Although, in healthy persons a HCMV infection is usually asymptomatic or developed mononucleosis like syndrome in rare case. It may develope severe diseases and cause of life threatening infection in person who absences of an effective immune responses, as in immunologically immature and immunocompromised individuals. Therefore, its impact has increased in organ allografting, immunosuppressive drug treatment and HIV infected patients. Moreover, HCMV is also a leading cause of pneumonia, hepatitis, encephalitis, esophagitis, colitis and retinitis in immunocompromised host (3, 7).

HCMV disease is a major cause of morbidity and mortality in immunocompromised patients. In these patients, early diagnosis of HCMV infection is

important since the development of HCMV disease may be prevented. There are several diagnostic laboratory methods to detect of HCMV infection, such as conventional cell culture, shell vial assay, serology diagnosis and antigenemia assay (8-10). However, these techniques are found to be low sensitivity and usually take a long times (10, 11). Detection of the HCMV genome by polymerase chain reaction (PCR) is higher sensitive than other methods (10, 11). Therefore, PCR based methods are suggested in order to increase the efficiency of HCMV diagnosis.

Although, typing of HCMV did not be divided in former times. Recently, HCMV genotypes can be differentiated, based on sequence variation of HCMV envelope glycoproteins, such as gB, gH, UL4, gN and gO (12-22). Sequence analysis and PCR-restriction fragment length polymorphism (PCR-RFLP) were now the methods of choice to type HCMV.

Glycoprotein B (gB) is one important component of the HCMV virion. The gB protein is not only necessary for virion penetration into cells and transmission of infected cells, but is also the major target for inducing neutralizing antibody and acts as target for cytotoxic T cell responses (23-26). Determination of gB genotype is based on the nucleotide sequence coding for highly variable region around the protease cleavage site, which is the neutralization related epitopes to recognize by murine monoclonal antibodies and human antisera (18). Nucleotide homology between clinical strains within each genotype was 98.6-99.9%, whereas homology between strains of differing groups was 88-95% (18). The distribution frequencies of gB genotypes in various body sites and in different groups of immunocompromised patients were analysed (4, 27-32). HCMV gB type 1 is less virulent than other gB genotype strains. gB type 2 and 3 in bone marrow transplant (BMT) recipients and HIV infected patients are associated with greater virulence, while the gB type 4 is rarely found in transplant recipients (4, 27-34). Moreover, coinfection with more than one HCMV gB genotype is often found in AIDS patients and most of the renal transplant recipients (30, 33, 35, 36). The gB genotype has been shown to correlate with cell tropism *in vivo* and may influence the virulence of HCMV. The gB type 2 and 3 can infect lymphocytes as well as granulocytes and monocytes, whereas gB type 1 can not infect T lymphocytes (28). A number of studies has attempted to correlate the distribution of HCMV genotype with the HCMV infection but it was still controversial. Although, the gB genotype was suggested to correlate with the clinical outcome of HCMV infection (4, 27, 30, 31, 37, 38), no association was found in some studies (29, 33, 35, 39-42).

Since HCMV is a significant cause of mortality and morbidity in patients, many antiviral drugs have been developed. Ganciclovir, foscanet and cidofovir are the three antiviral drugs currently used for treatment of systemic HCMV disease (3, 43).

Ganciclovir (GCV) is an acyclic analogue of thymidine active against all human herpesviruses. GCV must be converted intracellularly to the triphosphate, which is a potent inhibitor of viral DNA polymerase. GCV is efficient conversion to the monophosphate in HCMV infected cells (3). Although, HCMV does not synthesize a thymidine kinase, HCMV *UL97* open reading frame codes for a protein kinase homologue capable of phosphorylating GCV (44, 45). In severely immunocompromised patients with HCMV disease, prolong therapy is often necessary. A potential risk associated with prolonged used of antiviral components is emergence of resistant viruses (46). Several studies reveal that amino acid deletions or substitutions in conserved regions of the UL97 protein cause impaired GCV phosphorylation leading to GCV resistance of HCMV (44, 47-54).

Therefore, prevalence of the gB genotype may serve as a marker for pathogenicity and clinical outcome of HCMV while detection of *UL97* mutations may be useful to monitor antiviral therapy.

At present, no information of gB genotype among Thai patients is available. In this study, the prevalence of gB genotype and the attempt to determine GCV resistance of HCMV have been done.

CHAPTER II

OBJECTIVES

1. To study the prevalence of HCMV gB genotypes from clinical specimens of patients who participate in King Chulalongkorn Memorial Hospital during the year of 2000 to 2004.

2. To determine the mutation in the UL97 gene related to ganciclovir resistance in clinical specimens from patients who received GCV therapy.
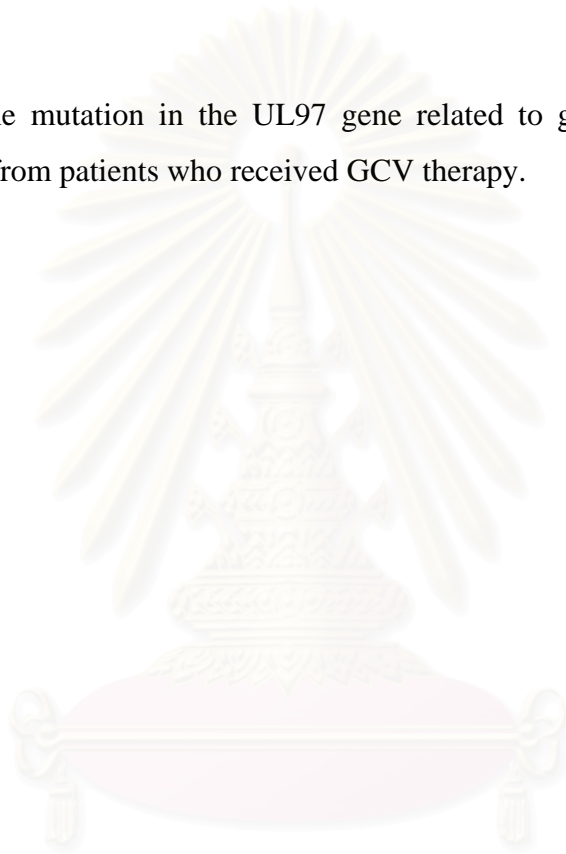
CHAPTER III

LITERATURE REVIEWS

I. Virology

HCMV is a member of the beta herpesvirinae, a subfamily of herpesviridae. HCMV has similar characteristics to other herpesviruses, such as virion and genome structure and the ability to establish persistent and latent infection. However, HCMV has distinct characteristics, including relatively restricted host range, long growth cycle and slow spread in cell culture. Classification of human herpesviruses is summarized in Table 1 (55). *In vivo*, HCMV can infect a large variety of cells, for example endothelial cells, monocytes/macrophages, epithelial cells, fibroblasts, etc. Whereas, the efficient replication *in vitro* is restricted to primary fibroblasts (1, 2).

HCMV has the largest genome among any of the herpesviruses, ranging from 230 to 240 kbp with a high guanine + cytosine (G+C) content (54 % - 59 %). HCMV is the only betaherpesvirus known to have a class E genome structure, including the existence of four genome isomers (Figure 1). In contrast, the genome of other betaherpesviruses is linear without repeat regions (Class F). Like that of herpes simplex virus, it contains an arrangement of unique long (UL), unique short (US) and repeated regions (Figure 1). Inversion of UL and US regions is immediated by inverted repeats. The repeated a sequence (*a, b, c*) occurs as a direct element at the termini and in the inverted (*a', b', c'*) orientation at the US/UL junction. In HCMV, the sequence carries the cleavage and packaging signal, called *pac-1* and *pac-2*, located on one site of the cleavage site (1). The AD169 laboratory strain is the only completely sequenced HCMV. Analysis of its 230 kbp genome has revealed that it encodes 225 open reading frames (ORFs) of approximately 100 or more polypeptides or proteins. These ORFs are designated sequentially according to their location within the unique and repeated regions (2).

The mature virions of HCMV range in size from 150 to 200 nm. Virion structure carries a large linear double-stranded DNA genome in a 100 nm diameter icosahedral capsid that is surrounded by a tegument or matrix. These components enclosed by a lipid bilayer containing a large number of viral glycoproteins. The phospholipid envelope contains 6 virus encoded glycoproteins, including gpUL55 (gB), gpUL73

(gN), gpUL74 (gO), gpUL75 (gH), gpUL100 (gM) and gpUL115 (gL). These glycoproteins play essential roles in virus entry into host cells, cell to cell spread and virion maturation (56).

Table 1. Classification of human herpesviruses.

Subfamily	Genus (-virus)	Example		Biological properties	
		Official name	Common name	Growth cycle and cytopathology	Latent infection
Alpha-herpesvirinae	Simplex	HHV1	HSV type 1	Short, cytolytic	Neurons
		HHV2	HSV type 2		
	Varicello	HHV3	Varicella-zoster virus		
Beta-herpesvirinae	Cytomegalo	HHV5	Cytomegalovirus	Long, cytomegalic	Glands, kidneys
	Roseolo	HHV6	Human herpesvirus 6	Long, lymphopoli-ferative	Lymphoid tissue
		HHV7	Human herpesvirus 7		
Gamma-herpesvirinae	Lymphocrypto	HHV4	Epstein-Barr virus	Variable, lymphopoli-ferative	Lymphoid tissue
	Rhadino	HHV8	Kaposi's sarcoma associated herpesvirus		
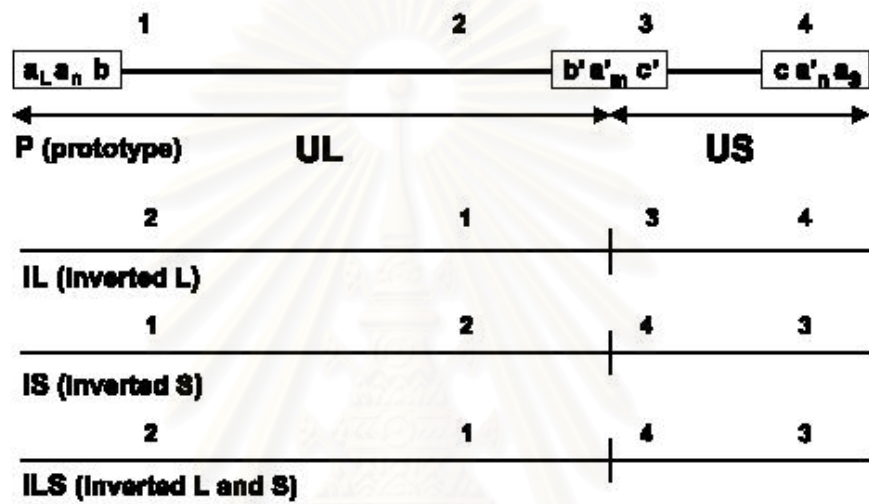
Figure 1. Structure of the four HCMV genome isomers (2).

II. HCMV glycoproteins

The envelope of HCMV containing host lipids and viral proteins, is derived from either the inner nuclear or cytoplasmic membranes. The early steps of virion attachment, fusion and penetration of the host cell have been assumed to be functions of viral envelope glycoproteins.

Several disulfide-linked glycoprotein complexes were identified in the envelope of HCMV (57). In 1988, Gretch et al. observed three distinct families of disulfide-linked glycoprotein complexes and designated glycoprotein complex gcI, gcII, and gcIII. They have designated these families gcI, gcII, and gcIII on the basis of their immunoprecipitations with monoclonal antibodies (58). Several ORFs coding for glycoproteins are expressed at immediate early time following infection. This later finding is in marked contrast to alphaherpesviruses and that HCMV glycoproteins could contribute to the transcriptional regulation of this virus. Over all, sequence analysis has predicted that clinical strains of HCMV may encode as many as 65 unique glycoproteins (59). Although glycoproteins have been analyzed, to date their functions in the replicative cycle of this virus is unknown.

Most envelope glycoproteins elicit strong host immune responses, including the production of virus neutralizing antibodies. Furthermore, the role of glycoproteins encoded in the US region of the viral genome have been shown to modulate the immunological recognition of virus infected cells (60). The function and selected characteristics of well studies HCMV glycoproteins are proposed by Britt et al., shown in Table 2 (56).

Table 2. HCMV glycoproteins (56).

Glycoprotein	Function	Host immunological response	
		Neutralizing antibody	**Cellular**
gB (UL55)	attachment, fusion	+	+
gH (UL75)	fusion	+	+
gL (UL115)	chaperon for gH	?	?
gM (UL100)	unknown	+	+
gp48 (UL4)	unknown	?	?

Glycoprotein B (gB)

gB is perhaps the most highly conserved envelope component of all members of the herpesvirus family. Furthermore, it has been estimated that gB represents greater than 50% of the protein mass of the envelope (56).

HCMV gB coding from ORF UL55 has been given various designations, including gB, gp55/116, gcI and gp58 (25). gB is the type I membrane glycoprotein composed of a transmembrane subunit, gp55, and the surface subunit, gp116. HCMV gB is made as a full-length glycoprotein of 906 amino acids. This polypeptide of the gB is glycosylated to a 150 kD precursor which is the major intracellular form of gB, and cleaved between residues 460 and 461 to generate the surface and transmembrane components. Moreover, gB forms homodimeric molecules linking by disulfide bonds to represent the gcI complex (56, 61).

HCMV gB is an abundant glycoprotein in the virion envelope that elicits neutralizing antibodies during human infection. The antibodies with neutralizing activity were studied for their effect on the attachment of virions to the cell surface, virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells (25, 62). An especially, domains within the transmembrane component of HCMV are responsible for the fusion activity of gB (25). Figure 2 represented current model of the antigenic and functional domains on the gB molecule (63).

N: Amino terminus aa 28-67   C: Carboxy terminus aa 476-645
M: Midregion aa 411-476   I: Intracellular aa 719-906
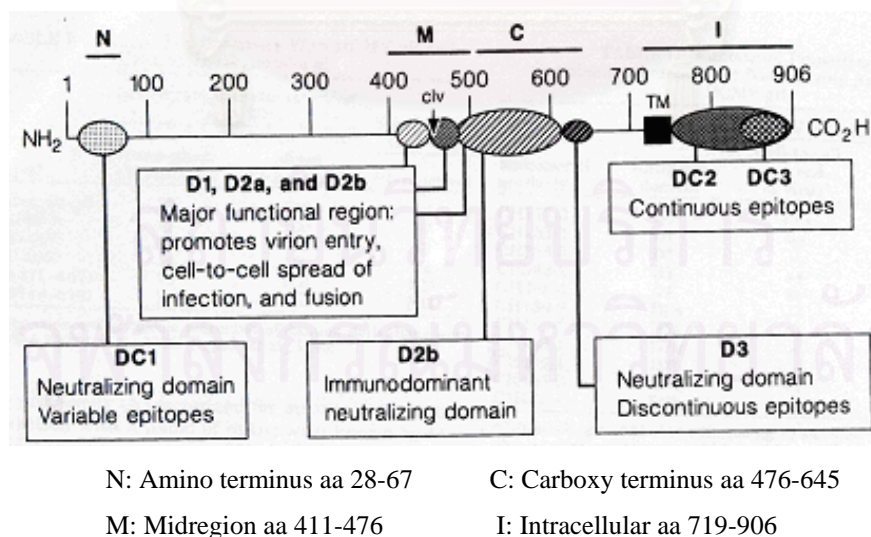
Figure 2. Topographic map of the antigenic and functional domains on HCMV (AD169) gB (63). Antigenic domains are shown as ellipses. Boxes show designations for the antigenic domains and list their functions. Shaded box shows the transmembrane domain.

gH-gL-gO complexes

HCMV attaches to the cell surface by binding gB to its receptor during the initial virus-cell interaction, however, final fusion of the viral envelope with the cell membrane to allow viral penetration required the heteroligomeric gH-gL-gO complex (2).

gH or UL75 is a component of the gcIII glycoprotein complex, which also includes UL74 (gO) and UL115 (gL) (58, 64-66). The gH component induces virus neutralizing antibody and facilitates penetration of HCMV into host cells (67-69). gL is necessary for transport of the gH glycoprotein from the nuclear membrane to the cell surface (70, 71). Similar to other herpesviruses, gH complex formation with gL is required for cell surface and virion location of the gH/gL complex (70-72). On the other hand, little is known about the function of gO. A role for gO in the fusion and entry processes has been suggested on the basis of properties of a gO deletion mutant virus (73). They found that the virus was severely impaired in growth kinetics as well as the process of cell to cell spread. However, the exact function of gO in the infection process has not been yet defined. In 2002, Paterson and colleague demonstrate that gO has a role in virus mediated cell fusion as shown by fusion inhibition using gO-specific antibodies in the fusion-form-without (FFWO) assay (66). Moreover, they indicated that gO plays a role in cellular spread mediated by membrane fusion.

gN-gM complexes

UL100 or gM is a component of the envelope gcII complex in association with UL73 or gN (74). gcII has been reported to consist of a heterogeneous family of glycoproteins with molecular mass between 39 and more than 200 kDa (75). HCMV gcII complex could bind heparin and suggested that this complex could have a possible role in virion attachment (76). The glycoprotein complex containing gM is an essential protein for *in vitro* replication of HCMV (73). gM associates with a second protein of 50 to 60 kDa through disulfide bonds and that both molecules represent major constituents of mature virions. The 50 to 60 kDa protein is encoded by the UL73 reading frame of HCMV AD169, which represents the gN homologue of herpesviruses. In the absence of gM, the UL73 gene product is a 18 kDa polypeptide. Whereas, it acquires complex modifications resulting in the formation of a 50 to 60 kDa a glycoprotein in the presence of gM. Moreover, that complex formation was required for the transport of both proteins from the endoplasmic reticulum (ER) to the golgi and *trans*-golgi components (77). Furthermore, the gM-gN complex of HCMV appeared to be antigenic in humans and was a target of antiviral antibody responses following natural infection.

III. Viral replication

Viral entry is the result of a cascade of interactions between viral and cellular proteins that culminate in fusion of the virion envelope with the cellular plasma membrane by a pH independent mechanism (2). During the initial virus-cell interaction, HCMV attaches to the cell surface by low affinity tethering interaction with heparan sulfate proteoglycans (HSPGs). The gM/gN heterodimer and gB both adhere to HSPGs following by their stable docking to epidermal growth factor receptor (EGFR) or with as yet unidentified receptor in hematopoietic cells, serves as a cellular receptor for HCMV. Ultimately, the virus fuses with the plasma membrane depositing virion components in the cytoplasm. The heteromeric envelope glycoprotein complex, gH/gL/gO, and gB are required for membrane fusion. The internalization of virion component is promoted by cellular integrins, serves as co-receptors. Interaction of HCMV glycoproteins with their receptors is enough to generate an intracellular signal transduction pathway, leading to alteration of cellular gene expression (78).

Following infection of a susceptible cell, the HCMV genome is expressed in a temporally coordinated and regulated cascade of transcriptional events. These occasions lead to synthesis of three categories of viral proteins described as immediate early (IE or α), early (E or β), and late (L or γ). The predicts of the IE genes are those the virus requires to take over control of host cell macromolecular synthesis, the E products are required to control production of viral progenies, while the L proteins form the structural components of the virions. The genome expression is termed cascade because the appearance of the earlier products is required for expression the later products. Thus as shown in Figure 3, IE proteins permit E mRNA synthesis, E proteins permit DNA replication which is followed by L mRNA synthesis (1, 2, 79). The IE and E genes are transcribed by host cell RNA polymerase II in the nucleus of infected cells. There is no evidence that this virus encodes its own RNA polymerase, although viral functions serve to modulate cellular transcription machinery during infection (1, 79).

As with the other herpesviruses, HCMV DNA is packaging into preformed capsid, which then acquires the tegument proteins and becomes enveloped (1, 80-82). Among the earliest events in the process of capsid formation, capsid proteins from their cytoplasmic site of synthesis move into the nucleus where assembly proceeds. Major capsid protein (MCP) interacts with the assembly protein precursor (pAP), which supplies the nuclear localization signals, that mediates MCP/pAP complex to pass through the nuclear pore (81, 82). Consequently, packaging of viral DNA into capsids

and addition of tegument proteins occurs within the nucleus, where nucleocapsids are transported out of the nucleus to the cytoplasm. In addition, after packaging of viral DNA into capsid, UL97 plays major role during viral replication manifests itself (83). UL97 is not critically required for very early events in viral replication for replication of viral DNA and packaging of this DNA into intranuclear capsids. However, UL97 is required at the stage of infection when nucleocapsids exit from the nucleus (nuclear egress). As generally accepted for herpesvirus, the earliest model for envelopment was suggested that the final envelopment takes place at the inner nuclear membrane (80, 84). However, Sanchez, V. et al. indicated that tegument protein pp150 remained within the cytoplasm throughout the replicative cycle of HCMV and accumulated in a stable form (85). Therefore, suggesting that HCMV cannot acquire its final envelope at the nuclear envelope. Thereafter, studies of assembly process suggested that primary envelopment of capsids occurred by budding at the inner leaflet of the nuclear membrane followed by a de-envelopment of capsids at the outer nuclear membrane. Subsequently, final tegumentation occurs in the cytoplasm and final envelopment of the tegumented nucleocapsid received by budding into vesicles of the *trans*-Golgi following with membranes containing processed envelope glycoproteins (82, 86, 87). Homman-Loudiyi, M. and colleague confirmed the previous proposed hypothesis that the envelopment of tegumented HCMV capsids occurs into cytoplasmic vacuoles, as shown in Figure 4 (87).

Progeny virus accumulates in the cytoplasm and infectious virus is released into the extracellular compartment beginning at 72 hr post infection. Even so, a substantial number of viral particles are still associated with the cell (2). HCMV infected cell culture produces three types of virus particles: infectious virions, non-infectious enveloped particles and dense bodies (DB). Non-infectious enveloped particles are defective viral particles composed of enveloped immature capsids that lack DNA but contain the viral assembly protein. DB are enveloped particles that lack an assembled nucleocapsid and viral DNA but contain several tegument proteins of which pp65 or lower matrix protein (1). HCMV infected cells may become enlarged (cytomegalia) and produce intranuclear inclusion with perinuclear halo as characteristic "owl's eye".
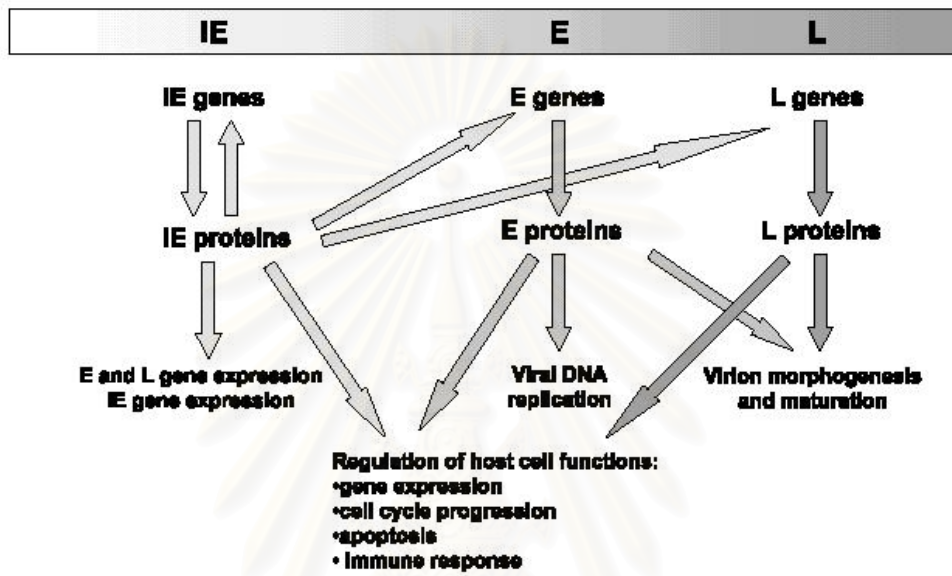
Figure 3. HCMV gene expression and viral gene product functions during productive infection (2).
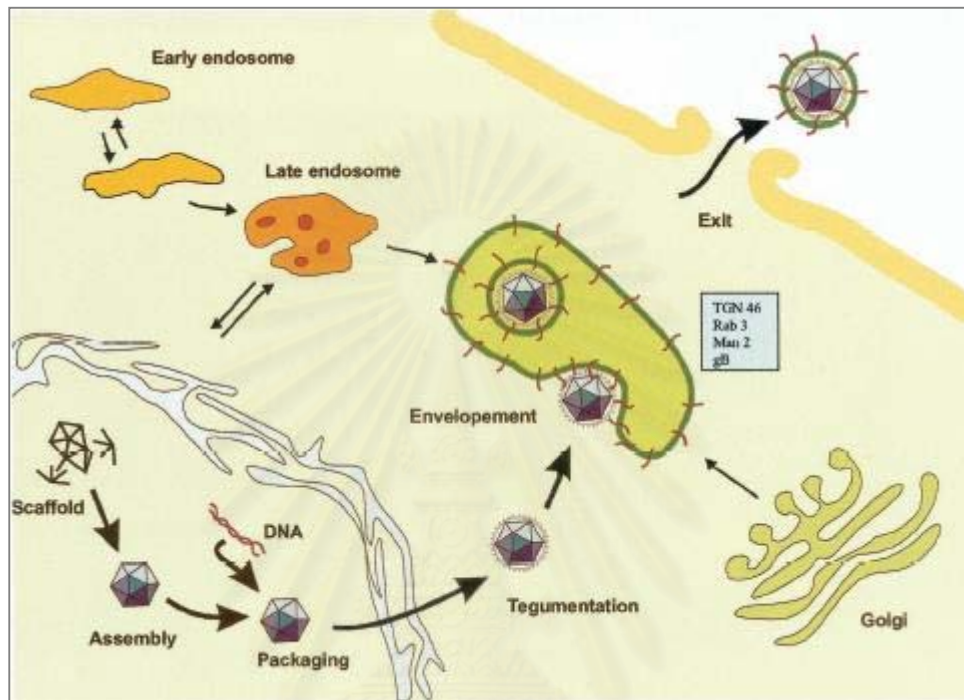
Figure 4. Model of the HCMV envelopment and exit pathway (87). Naked nucleocapsids, formed in the nucleus, transverse the nuclear membrane and become tegumented through the exit pathway by an unknown process. Viral glycoproteins are released from the Golgi apparatus within small transport vacuoles. The tegumented HCMV nucleocapsids become enveloped when budding into the vacuoles, acquiring the vacuole membrane as their envelope. The vacuoles are transported to the plasma membrane, and virus particles are subsequently released upon fusion of the vacuoles with the cellular plasma membrane.

IV. Pathogenesis and disease of HCMV infection

HCMV is a lytic virus, as evidenced by its cytopathic effect in tissue culture cells and by the presence of cytomegalic inclusion bearing cells in involved tissue. Several *in vitro* properties of the virus including slow replication, restricted cell tropism, limited cell to cell spread and especially, high titer replication of HCMV in cell culture is restricted to primary human fibroblasts (88). During acute infection *in vivo*, HCMV nucleic acids and antigens have been found in various organs, such as in the liver, lung, gastrointestinal tract, retina, central nervous system, kidney and peripheral leukocytes.

Studies on HCMV cell tropism *in vivo* revealed three characteristics: 1) ubiquitously distributed cell types, such as epithelial cells, endothelial cells and fibroblasts are the major targets of HCMV infection; 2) leukocytes circulating in the peripheral blood are susceptible to the virus, and 3) specialized parenchymal cells such as smooth muscle cells in the gastrointestinal tract and hepatocytes can also be infected (89, 90). Epithelial cells seem to be highly susceptible to HCMV infection (89). Since the epithelial layers of the respiratory tract, of the gastrointestinal tract, and of the genitourinary tract build the interface between the organism and its environment, they are important sites for viral entry into the host as well as for the release of virus via body excretion (90). Endothelial cells and monocytes /macrophages may play a crucial role in the haematogenous spread of HCMV. In contrast, fibroblasts, smooth muscle cells and epithelial cells may form the cell populations important for the multiplication and spread of the virus in infected tissues (89, 91). Clinical observations suggest that the appearance of circulating infected endothelial cells in the peripheral blood was associated with increased risk of symptomatic disease (92).

Then, after HCMV infection, the virus spreads locally to lymphoid tissue and the systemically in circulating lymphocytes and monocytes to involve the lymph node and spleen. Then, the infection localizes in the ductal epithelial cells, such as salivary glands, kidney tubules, cervix, testes, epididymis, etc (Figure 5). Therefore, the virus may shed in oropharyngeal secretions, breast milk, urine, semen and cervical secretion often for years (2, 5). After primary infection, it may involve a combination of latency with integration of viral genome into leukocytes and chronic infection with production of infective virions.
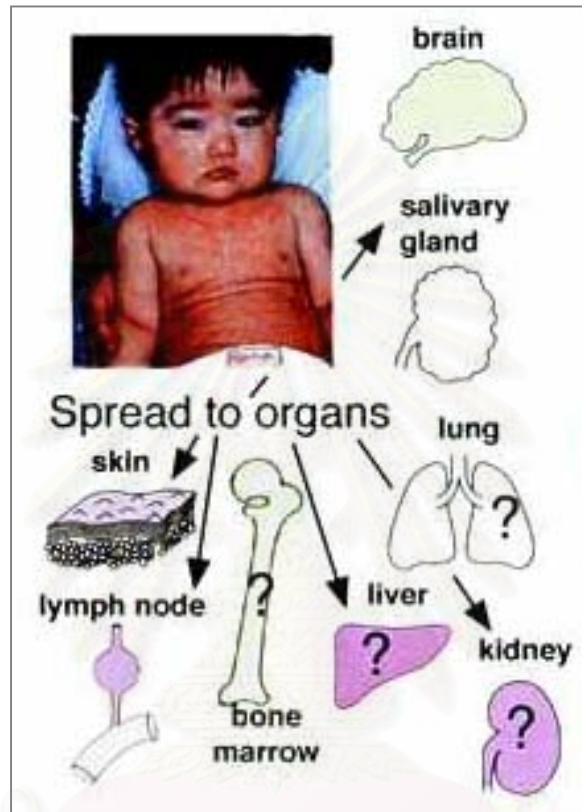
Figure 5. Targets for HCMV replication and its localization.

As a rule, the cause of infection in healthy persons with HCMV is usually asymptomatic or mild. Reactivation is clinically silent in immunocompetent individuals, although infectious virus might be shaded by various organ excretions (5, 93, 94). Nevertheless, HCMV leads to serious manifestation, and in some cases the disease may be fatal, as in immunocompromised individuals. HCMV infection may also be the cause of fever, leukopenia, hepatitis, nephritis, pneumonia, arthralgias, esophagitis, gastritis, enteritis, colitis, encephalopathy or encephalitis and retinitis, as has been found in immunocompromised hosts (3, 7, 95). Even so, there was no evidence of HCMV activation after post transfusion from HCMV seropositive donor (96).

In transplant patients, the degree of immunosuppression in the recipient is closely linked with the probability of a symptomatic infection. Three major epidemiologic patterns of HCMV infection are found in organ transplant recipients: primary HCMV infection, reactivation HCMV infection, and superinfection. Primary HCMV infection occurs when an individual who is HCMV seronegative becomes infected with virus carried latently in cells from a seropositive donor. While, the transplant recipient who has been infected with HCMV previously undergoes reactivation of endogenous latent virus after transplantation. Furthermore, HCMV seropositive recipient may represent superinfections by a second strain when that received allograft from HCMV seropositive donor (97). In addition, HCMV is associated with an increased risk of acute graft rejection. This has been shown for recipients of heart, lung, kidney, and liver transplants (7). On the other hand, HCMV infection did not increase the risk of either acute and chronic graft versus host disease (95).

HCMV is an important opportunistic viral infection in HIV infected persons. It frequently produces severe disease even with recurrent infection in seropositive individuals. This is not surprising because HIV can produce functional defects in a number of cells responsible for cell mediated immunity, especially the CD4+ T cell. Indeed, specific defects in cell mediated immunity against HCMV have been defected in AIDS patients although their CD4+ T cells were reconstituted (98-101). One of the most serious HCMV associated diseases in AIDS patients is the HCMV retinitis that leads to progressive loss of vision and blindness (3). The incidence of HCMV retinitis is 10%-40% in AIDS patients (102). Development of HCMV disease occurs late in HIV infection and is mostly seen in patients with CD4+ T cell counts of lower than 100×10^3 cells/ml (103).

V. Persistence and latency

Like all herpesviruses, HCMV remains with its host for life after primary infection that is characterized by persistence of the viral genome without the production of infectious virus. The definition of viral latency implies that functional viral genome is retrived in the host after resolution of productive primary infection, and that certain signaling events can reactivate productive cycle gene expression, which results in recurrence of infectious virus and revival of clinical symptom (104). Most individuals become infected with HCMV early in life, and depending on the geographic location, between 60 % and 100 % of adults are carriers of the virus (88). Reactivation of HCMV from latency results in serious morbidity and mortality in immunocompromised patients. Although, the site of HCMV latency is unknown, blood cells have been implicated as a viral reservoir. Dissemination of the virus in the blood occurs mainly via leukocytes, which are the major source of HCMV spread (105). Sodenberg-Naucler et al demonstrated that PBMCs harbor latent HCMV, which reactivates in a myeloid lineage cell upon allogeneic stimulation (106). In addition, HCMV infection of endothelial cells and monocyte-derived macrophages (MDMs) plays an important role in the establishment of latency and persistence. Thus, macrophage differentiation is a prerequisite for productive HCMV infection (90, 107). Furthermore, the immune privileged retinal pigment epithelium is a major site of persistent HCMV (102). There is considerable uncertainly as to the nature of latent infection: whether the IE genes are expressed in latently infected cells. The IE proteins are transcriptional transactivators which are required for induction of early gene expression and progression to productive infection. IE gene expression is extremely low in latently infected cells and is required for lytic viral replication, it is likely that induction of IE gene expression is the first step in reactivation of the HCMV (102, 104, 108). Accordingly, detection of IE1 transcripts in latently infected spleens and lungs was taken for an evidence for recurrent or chronic infection (109, 110). Latently infected monocytes in the peripheral blood are activated by an immune response. During this response, CD4+ and CD8+ lymphocytes, that is necessary for induction of the allo-MDM phenotype required for reactivation of latent virus (Figure 6) (107, 111). The mechanism by which HCMV reactivates from latency has not been well understood because of its strict species specificity. Like HCMV, murine CMV (MCMV) has been used as a model for HCMV infection and disease (104, 108). There are three models for reactivation from latency that have been reported: (1) reactivation is due to immunosuppression, (2) reactivation is induced by cellular

differentiation, and (3) reactivation is induced by the allogeneic response to the transplanted organ (108).

VI. Immune response to HCMV infection

HCMV infection is kept under control by the immune system. Nevertheless, total HCMV clearance is rarely achieved and the viral genome remains at selected sites in a latent state. Since severe infection is usually restricted to individuals with impaired cell mediated immunity, it is evident that this arm of the immune response provides the most protection. Even so, the humoral system plays a supportive role to keep HCMV loads below critical thresholds. Neutralizing antibodies (NAb) do not play a primary role in recovery because infection is generally cleared before any obvious increase in antibody levels. While, cell mediated immunity plays the important roles to control of HCMV infection. Although NK cells are also important in controlling virus until specific immunity develops, their activities are insufficient by themselves to eliminate virus. However, CMV reactive cytotoxic T cells (CTLs) correlates with protection from HCMV infection (1, 2).

In studies of HCMV infection, most have emphasized the importance of cellular immune responses, but high levels of virus NAb have been associated also with a favorable clinical cause in HIV-1 infected persons (112). A role of NAb is also indicated by a report showing that titers are lower in mothers who transmit HCMV to their fetus than in mothers who do not transmit the infection (113). HCMV glycoproteins contain major antigenic determinants of the virus. A major fraction of NAb in the sera from HCMV infected patients was directed to HCMV gB (114, 115).
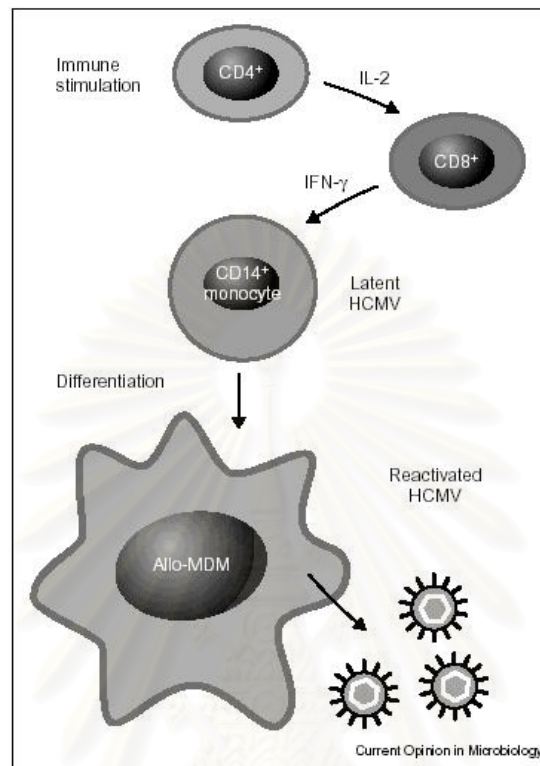
Figure 6. Model for reactivation of latent HCMV *in vivo* (107). An allogeneic reaction or a response against pathogens activates the immune response. This activation event induces production of IL-2 by CD4+ lymphocytes, stimulating IFN-γ secretion by CD8+ lymphocytes. In combination with other factors, IFN-γ induces CD14+ monocytes to differentiate into Allo-MDMs, reactivating latent virus and resulting in the production of infectious virus.

The antibody to neutralizing epitopes, not the antibody to epitopes on the whole HCMV gB, could be the main factor in disease prevention. Three antibody binding sites have been mapped on the gB molecule, only AD1 (codons 552-635) and siteII of AD-2 (condons 67-86) that were continuous neutralizing epitopes have been shown to induce virus-NAb (116-118). In addition, AD-2 specific antibody efficiently reduces the virion-mediated fusion (119). Besides that gB also contained 4 neutralizing domain discontinuous epitopes, which lined in the midregion (codons 411-476) and a part of carboxy-terminal half (codons 476-618) (118). The midregion of gB between residues 447 and 476, and residues 476-618 from the carboxy-terminal half were necessary epitopes requiring for the assembly of complement-independent NAb (63, 118). Although, humoral immunity, especially neutralizing antibody alone, could not prevent HCMV disease progression, there must have been existing prevention factors in the sera of healthy persons (120). Although the presence of NAb has been persisted, it is well known that virus shedding, especially in the immunocompromised host is demonstrated. Rasmussen, L. and Cowen, C.M. revealed that HIV infected patients had rising NAb titers despite recovery of HCMV at various times during monitoring (121).

HCMV specific CTL response was required for recovery from HCMV infection. Further studies showed that CD8+ cytotoxic T cells are primary importance in the prevention of recurrence (122, 123). Moreover, HCMV specific CTL killed virus infected cells before viral DNA replication, suggesting that they recognized predominantly non structural viral proteins. Borysiewicz et al. examined HCMV specific CTL for envelope gB and 72-kD IE protein from asymptomatic persistently HCMV infected individual. The relative number of HCMV 72-kD IE protein specific CTL was greater than gB specific CTL. CTL with this specificity may be important in maintaining the normal virus/host equilibrium (24). Besides MHC class I restricted responses, in normal asymptomatic subjects the MHC class II restricted response against gB may predominate (26). Moreover, HCMV specific T cell responses may involve an age dependent susceptibility because early childhood HCMV infections are characterized by much more prolong than is observed in adults (124, 125). Recent reports revealed that HCMV specific CD8+ T cell responses in asymptomatic children with active infection were not different from adults with recent or long term infection in frequency and function analyses (126-128). While, HCMV specific CD4+ T cells that produced IFN-γ of immunocompetent young children accumulated markedly fewer than adults (128). During HCMV infection, cellular gene expression would be altered since

many of the cellular factors that become activated in responses to HCMV are transcriptional regulators. The expression of hundreds of cellular genes is altered in cell that exposed to HCMV particles (129). Previous studies have shown that HCMV is a potent elicitor of interferon stimulating gene (ISG) expression. Member of ISG family is most dramatically up regulated in response to HCMV. Envelope gB from HCMV is a viral structural component that can directly induce transcription of ISGs. Especially, the region of gB from residues 461 to 750 is important for initiation of the antiviral responses (130).

Both humoral and cell mediated immunity participate in effective protection against HCMV disease. The attenuated Towne vaccine has been tested both in healthy volunteers and in transplant recipients. It induced neutralizing Abs and CTL response and reduced HCMV disease in seronegative renal transplant recipients, but did not prevent infection (131, 132). In addition, several approaches have been used to develop an effective and safe subunit HCMV vaccine. More recent interest in subunit vaccines for the prevention of HCMV disease has focus on the production of HCMV proteins from recombinant expression system. A large effort has been directed toward the major envelope glycoprotein, gB. Because HCMV gB is the major target for HCMV NAb, it is a prime candidate for a HCMV vaccine (133-136). Repeated inoculations of humans with gB combined with MF59 adjuvant have induced significant antibody responses (134), and recombinant adenovirus and canarypox viruses expressing gB proteins induced antibody and CTL responses (133, 135). In addition, immunization with plasmid VR-pp65 expressing the HCMV phosphoprotein 65 (pp65) induced high levels of CTL in mice (136).

VII. Anti-HCMV drugs

Different antiviral agents have been used in attempts to treat established cytomegalic diseases. The preparation has included leukocyte interferon, interferon stimulators, transfer factor and nucleoside drugs [iododeoxyuridine, fluorodeoxyuridine, cytocine arabinoside, adenine arabinoside (ara-A, vidarabine)]. Each of these regimens had little of no clinical benefit and each had unacceptable degrees of toxicity. Therefore, none of these regimens proved to be clinically useful (88, 137). To date, the nucleoside analog ganciclovir [GCV; 9-(1, 3-dihydroxy-2-proproxymethyl) guanine], the nucleotide analog cidofovir [CDV;(s)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine], the pyrophosphate analog foscanet (PFA), and formivirsen have been licensed for serious or life-threatening HCMV infections in immunocompromised individuals (2, 3). The structure of GCV, CDV, PFA and formivirsen were showed in Figure 7. All of them are inhibitors of the HCMV DNA polymerase with the exception of formivirsen, is an antisense oligonucleotide inhibiting the essential IE viral events by blocking the HCMV IE mRNA. For treatment of HCMV associated diseases and preemptive treatment of infection, the nucleoside analog GCV is the most widely used of these drugs.

GCV is an acyclic analog of thymidine active against all human herpesviruses. It is a competitive inhibitor of viral DNA polymerase (UL54) by residing in its nucleic acid chain-terminating activity (88). GCV must be converted intracellulary to the triphosphate. The drug is phosphorylated to the monophosphate by viral thymidine kinase, and then further phosphorylated to the triphosphate by cellular kinase. Then, the GCV-triphosphate incorporated into the viral DNA by the DNA polymerase resulting in inhibition of virus replication (3, 138). Even though, GCV is efficiant conversion to the monophosphate in HCMV infected cells, HCMV does not synthesize a thymidine kinase. There is report revealed that the HCMV UL97 protein shares homologies with protein kinase and bacterial phosphotransferase (139). In 1992, several reports revealed that HCMV UL97 open reading frame codes for a protein kinase homologue capable of phosphorylating GCV, as shown in Figure 8 (44, 45). According to different clinical setting, different treatment protocols were developed: 1) symptomatic or differed for patients with overt HCMV related disease; 2) preemptive for patients with active HCMV infection at risk of developing HCMV disease; or 3) prophylactic for patients at risk for HCMV disease in the absence of active HCMV infection. These treatment protocols have been applied to patients with AIDS, solid-organ transplant recipients and

hematopoietic stem cell transplant recipients (140). It is administered by intravenous infusion, and is usually the front-line drug for treatment of HCMV infection. An oral formulation that is effective for prophylaxis in immunocompromised patients has been approved (141, 142). However, its side effects include potentially dose limiting neutropenia and the emergence of GCV resistant strains during prolonged maintenance therapy (2, 3, 46). During the last decade, GCV resistant HCMV isolates have been recovered mainly from AIDS patient suffering from HCMV retinitis or gastrointestinal disorders, and transplant population (46, 52, 54, 143-147). Acquisition of mutations in UL97 phosphotransferase appears to be a crucial step in the selection of GCV resistant HCMV strains. HCMV strains with amino acid substitutions or short deletions in key regions (domains VI, VIII, IX) of the viral enzyme have been proven to be highly resistant to GCV (Table 3) (44, 47, 49-54, 148). All of these resistance mutations map to one of three sites: codon 460, 520, or 590-607 (Figure 9). It is now possible to analyze these regions using DNA extracted directly from clinical specimens. The mutations of UL97 could be detected by restriction enzyme polymorphisms (47, 52, 53, 149-153) or by DNA nucleotide sequencing (46, 47, 49, 52-54, 140, 145-147, 150, 153, 154). The latter is now the method of choice because of advances in sequencing technology and because not all mutations affect restriction enzyme recognition sequences. In addition to UL97, mutations in UL54 impact GCV susceptibility (140, 141, 155, 156). However, mutations in UL54 are less common and have been reported only in patients harboring GCV resistant UL97 mutant strains maintained on GCV treatment (156, 157). In summary, GCV resistance results from mutations in either UL97 or UL54 or both. Base line sequences of the UL97 are available for GCV sensitive HCMV clinical isolates. These sequences provide data for the normal sequence variability (polymorphism), which is not associated with drug resistance. However, definitive proof that a newly identified mutation confers resistance requires transfer of the mutation to a well-characterized drug sensitive HCMV strain (44, 47, 51, 154).
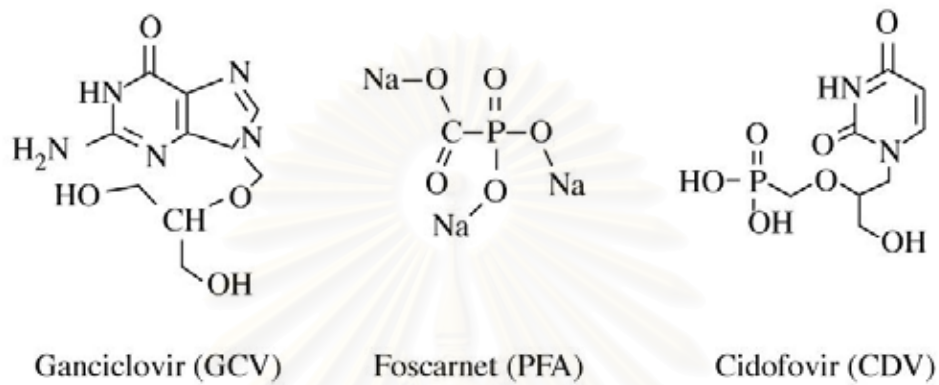
5'-GCGTTTGCTCTTCTTCTTGCG-3'

Fomivirsen

Figure 7. The structure of HCMV drugs (141, 158).
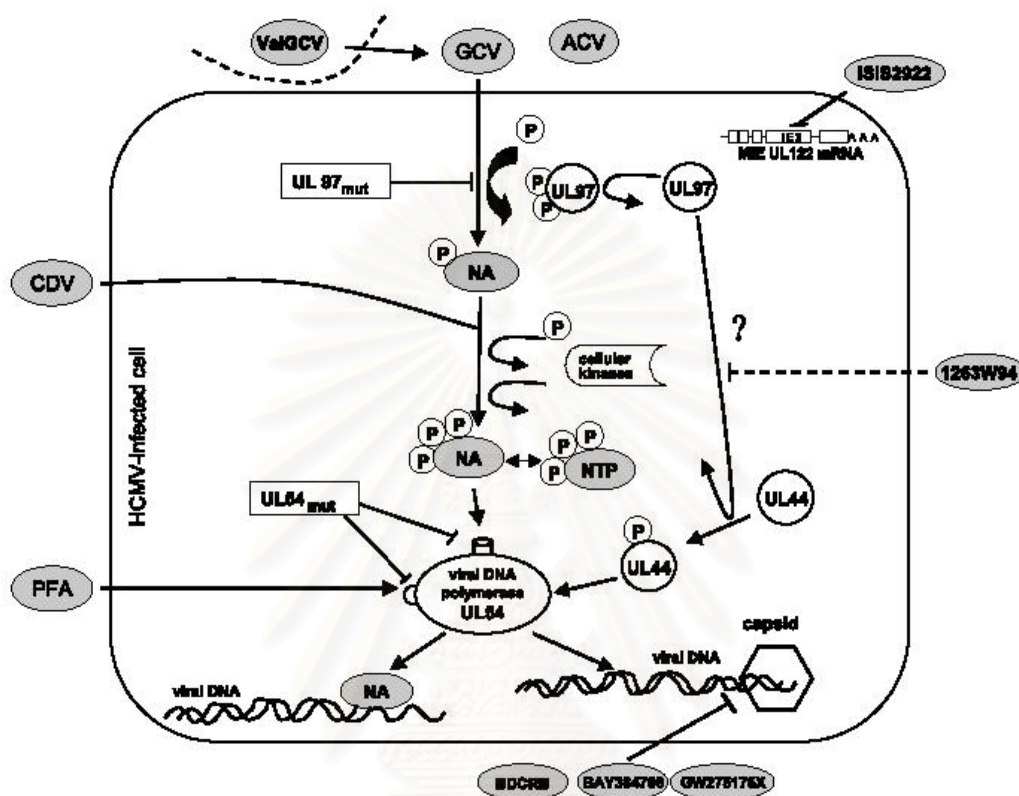
Figure 8. Mechanism of action of anti-HCMV compound (158). The nucleoside analogue (NA) GCV is monophosphorylated by the viral encoded protein UL97 (pUL97). After further phosphorylation, the GCV triphosphate competes with the natural nucleoside triphosphate GTP at the viral polymerase and is incorporated into the viral DNA. Besides GCV, pUL97 is able to direct the phosphorylation of the viral DNA polymerase processivity factor pUL44. CDV does not require monophosphorylation by pUL97. PFA directly inhibits the polymerase. Mutation in the pUL97 can confer resistance to GCV, however, these strains are still sensitive to CDV and PFA. Different mutation in the viral polymerase can confer resistance to CDV (often with a cross-resistance to GCV) or PFA.

Table 3. UL97 mutations associated with HCMV GCV resistance.

Codon	Amino acid wild type	Amino acid mutation
460	Met (M)	Val (V), Ile (I)
510	Asn (N)	Ser (S)
520	His (H)	Gln (Q), Glu (E)*, Thr (T)*
590*	Ala (A)	Thr (T)
591	Ala (A)	Val (V), Asp (D)*
592	Cys (C)	Gly (G)
594	Ala (A)	Val (V), Thr (T), Trp (W), Pro (P)
595	Leu (L)	Phe (F), Ser (S), Trp (W), Thr (T)*
596	Glu (E)	Gly, (G), Asp (D)*
597*	Asn (N)	Ile (I)
598	Gly (G)	Ser (S), Val (V)*
599*	Lys (K)	Met (M)
600	Leu (L)	Deletion
601	Thr (T)	Met (M)
603	Cys (C)	Trp (W), Tyr (Y)*
606*	Ala (A)	Asp (D)
607	Cys (C)	Thr (T), Tyr (Y)
590-593		Deletion
591-594		Deletion
595-603		Deletion
591-607		Deletion
597-603		Deletion
601-602		Deletion

* Mutation coffering GCV resistance revealed by Arens, M.Q., Department of Pediatrics, Washington University School of Medicine (personal communication).
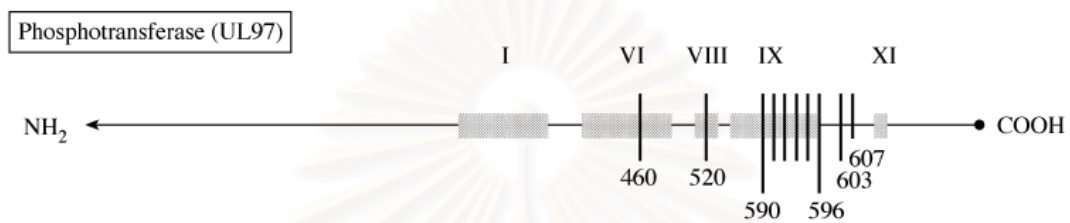
Figure 9. Schematic of HCMV phosphotransferase (encoded by UL97). UL97 codons mutated in GCV-resistant HCMV strains are shown by vertical solid bars (141).

VIII. Epidemiology

Humans are believed to be the only reservoir for HCMV. It was the most important cause of morbidity among immunocompromised patients and transfusion acquired infection (especially in small premature newborns), and congenital viral infection (159). Although, HCMV infection is self-limited, viral excretion can continue for extended periods of time and the virus persists throughout life. HCMV transmission occurs by both vertically and horizontally. Virus can appear following primary infection, reinfection or reactivation.

HCMV is a widespread pathogen responsible for HCMV infection between 50% and 100 % of normal population worldwide (1). In general, the prevalence of HCMV is lowest in developed countries and in high socioeconomic classes, and highest in poor countries and people with a low socioeconomic status (159, 160). Moreover, infection increases when people live in crowded, unhygienic condition besides in socially disadvantaged countries (2, 159).

Breast feeding is the most common route of transmission. Consumption of infected breast milk led to infection of 69 % of the infants, although there was some milk secretory immune response to this virus (5). The precise route of infection throughout childhood is not known, although close contact is required. After infancy in most developed countries, infection rates increase slowly until the age of entry into school, at which time they rise more rapidly, increased from 10 % to >80 % within one year (161). Since the incidence of infection in the day care centers, high rates of salivary shedding of HCMV and frequent exchange of oral secretions, it is likely that transfer of virus occurs through saliva on hands or toys (162). The incidence of congenital infection is highest in poor communities, since most women are infected before puberty. In the United States and Western Europe, seroprevalence rates in young women of childbearing age range from 40 % for women of middle to upper socioeconomic status to 83 % for women of lower one. In contrast, in several areas of Africa, Southern America and Asia infection rates of 90 % to 100 % have been recorded (160). Recently, India conducted to estimate the seroprevalence of voluntary blood donors in Delhi, 95 % were seropositive (163). HCMV infection is also very common in Thailand since most of Thai population have been infected with HCMV early of their life (164-166). Furthermore, the seroprevalence in female especially pregnant women are more predominantly than male (167-169).

IX. Genotyping of HCMV

Previously, categorization of HCMV had never been performed. Since HCMV genomic variants have been predicted (170-172), by this time HCMV genotypes can be generated for a variety of genes. Typing of HCMV based on sequence polymorphism of its envelope glycoproteins, such as gB, gH, UL4, gN, gL and gO (12-15, 17-22). HCMV envelope glycoproteins have been focused for genotyping arising from they are target for NAbs and are involved in virus entry and cell to cell spread (25). The variant group in each gene can be determined simply by restriction analysis of a small target sequence amplified from HCMV genomic DNA.

The HCMV genes encoding gB and gH are the most widely studies their polymorphism. As far as gB is concerned, four genotypes (gB1, gB2, gB3, and gB4) have been observed (4, 18, 21, 28, 36, 173). While, only two different genomic variants of gH (gH1 and gH2) have been identified so far (21). The other HCMV polymorphic surface glycoproteins have recently been discovered, including four UL4 genotypes (UL4-1, UL4-2, UL4-3A, and UL4-3B), seven gN genotypes (gN1, gN2, gN3a, gN3b, gN4a, gN4b, and gN4c), four gL genotypes (gL1, gL2, gL3, and gL4), and seven gO genotypes (gO1a, gO1b, gO1c, gO2a, gO2b, gO3, and gO4) (13, 15, 17, 22). The HCMV genes have been determined for their genetic linkage. Fries et al. revealed that isolates of gB1 were more commonly found to be of gH2, whereas the other gB genotypes were more commonly linked to gH1 (4). On the other hand, HCMV gB genotypes were neither associated with UL4 genotypes nor gN genotypes (16, 22). Recently, several studies showed that both gH and gN were correlated with gO variants (13, 17).

Since the existence of distinct gB genotypes have been observed, the variations of inter- and intra-gB genotype were evaluated among strains of HCMV. It revealed that nucleotide homology within each genotype was 98.6% to 99.9%, whereas homology between strains of differing genotype was 88% to 95% (18). Furthermore, many studies have attempted to find a correlation between the gB genotype and the occurrence of HCMV associated disease in immunocompromised patients. By this time, it has been still controversial. Although, the gB genotype was demonstrated to correlate with the clinical outcome of HCMV infection (4, 27, 30, 31, 36-38), no association was found in some studies (29, 33, 35, 39-42). It has been shown that gB genotype occurs in different frequency in certain populations, as shown in Table 4. Various reports have confirmed different prevalence. HCMV gB2 was found more frequent than the other genotypes in

HIV infected patients with symptomatic HCMV disease (27, 29-31, 41, 42, 174, 175). In addition, an association between gB2 and high risk of HCMV retinitis has been reported (37); it is not only a major determinant of retinitis pathogenicity but also appears to be highly prevalent among HIV infected patients (175). However, there was evidence revealed that gB1 is the most frequent in AIDS patients (39). Out of HIV infected patients, gB1 was the most prevalent genotype detected in both allograft transplant recipients and immunocompetent persons (4, 27, 33, 34, 38, 42, 176-179). In contrast, some studies showed that gB2 was exhibited in bone marrow transplant (BMT) recipients with HCMV disease and renal transplant recipients more frequently (32, 34, 40). Besides that, gB3 and gB4 may be associated with a reduced hazard of grade II to IV acute graft versus host disease (GVHD) (38). In addition, various studies demonstrated different distributions of gB genotypes in different body fluid in immunocompromised individuals (27, 28, 31, 173). Meyer-Konig et al. showed that HCMV gB1 did not infect T lymphocytes, while gB2 and gB3 infected lymphocytes as well as granulocytes and monocytes (28). In HIV infected patients, gB4 was found significantly more often in semen than in leukocytes (27). The frequency of gB2 was the most prevalent genotype in salivary and CSF, whereas gB3 was frequently found in sera (31, 173). Even so, there is contrary result that could not determined the relationship between gB genotypes and tropism for different body sites in the same patient with renal transplantation (32). Excluding of gB1 to 4 genotype, mixtures of gB genotypes were determined either different samples from the same patient or a single sample. The presence of multiple gB genotypes was the most incident in transplant recipients (35, 36). In addition, Coaquette et al. provides new and provocative evidence that HCMV infection with mixed gB genotypes in immunocompromised patients is associated with increased progression of HCMV disease, higher HCMV load, and an increase in the number of graft rejection episodes (36).

Table 4. Previous study population and geographical distribution of HCMV gB genotype.

Area	Country	Study population	gB genotype (%)		Specimen	Year (Reference)
America	Brazil	161 samples (41 hemato/oncology, 30 AIDS, 30 alcoholics, 30 blood donor, 30 pregnant women)	gB2 (73) gB1 (16) gB3 (11)		Plasma	2004 (174)
	Brazil	26 samples BMT[a]	gB2 (57.7) gB3 (23.0) gB1 (15.4) gB4 (3.9)		PBL	2003 (180)
	Brazil	20 Renal Tx[b]	gB2 (40) gB1 (30) gB3 (25) gB4 (5)		Blood Saliva Urine	2003 (32)
	Brazil	42 HIV	gB1 (42.8) gB2 (23.8) gB3 (16.6) gB4 (16.6)		CSF Urine Saliva	2003 (39)
	Canada	50 Solid organ Tx	gB1 (38) gB3 (24) gB2 (18) mixed (16) gB4 (4)		Leukocytes	2003 (179)
	U.S.A.	31 HIV (15 w[c] CMV retinitis, 16 w/o[d] retinitis)	w CMV retinitis gB2 (53) gB1 (20) gB3 (13) gB4 (13)	w/o CMV retinitis gB2 (50) gB1 (44) gB1+2 (6)	Plasma	2002 (175)
	Brazil	34 Renal Tx	Mixed (70.6) gB1-4 (29.4)		PBL Urine	2000 (35)
	U.S.A	120 HIV w CMV retinitis	gB2 (43) gB4 (21) gB1 (19) gB3 (12)		Vitreous	1998 (41)

Area	Country	Study population	gB genotype (%)		Specimen	Year (Reference)
America	U.S.A	281 BMT	gB1 (48.4) gB3 (24.6) gB2 (16.4) gB4 (8.2)		-	1997 (38)
	U.S.A	HIV Allograft recipients	urine: gB1 (39) gB2 (30) gB3 (17) gB4 (13)	leukocytes: gB3 (43) gB1 (36) gB2 (21)	Urine Blood Semen	1997 (27)
	U.S.A	44 HIV w CMV retinitis	gB2 (41) gB1 (27) gB3 (16) gB4 (16)		PBL	1996 (37)
	U.S.A	128 BMT (52 with fatal, 60 with non fatal)	Fatal: gB1 (38) gB3 (25) gB2 (15) gB4 (14) mixed (8)	Non fatal: gB1 (67) gB3 (18) gB2 (5) gB4 (5) mixed (5)	Blood Urine Aqueous humor	1994 (4)
Europe	Austria	42 CMV seropositive with GBS[e]	gB1 (88) gB3 (8) gB2 (4)		CSF	2004 (176)
	France	64 Immunocompromised patients (organ Tx, lymphoma, leukemia etc.)	gB1 (28.9) gB3 (23.7) gB2+gB3 (12.4) gB1+gB2+gB3 (3.1) gB4 (2.0) gB1+gB2+gB3+gB4 (1.0)		Plasma Whole blood WBC, BAL Urine	2004 (36)
	Spain	66 AIDS	gB3 (42.5, serum) gB2 (38.5, CSF) gB1+3 (27.5, semen)		Serum CSF	2003 (173)
	Italy	23 BMT 11 Renal Tx 8 HIV 23 congenital 17 immunocompetent	BMT: gB3 (52) gB2 (35) gB1 (13) congenital: gB1 (39.1) gB2 (26.1) gB3 (17.4) gB4 (17.4)	Renal Tx: gB3 (54.5) gB2 (36.4) gB1 (12.5) Immunocompetent: gB2 (58.8) gB3 (35.3) gB1 (5.9) HIV: gB2 (50.0) gB3 (37.5) gB1 (12..5)	Blood Urine	2003 (42)

Area	Country	Study population	gB genotype (%)		Specimen	Year (Reference)
Europe	-	37 HIV	gB2 (71.4) gB1 (7.14) gB3 (7.14) gB4 (3.57) gB2+other (3.57) gB3+other (3.57) gB1+3+4 (3.57)		Saliva	2001 (31)
	Germany	76 Organ Tx 47 BMT	gB2 (32.74) gB1 (31.86) gB3 (23.89) mixed (7.08) gB4 (4.42)		PBL	1998 (28)
	Germany	60 Kidney Tx 47 BMT	BMT gB1 (43) gB2 (34) gB3 (13) mixed (11)	Kidney Tx gB2 (30) gB3 (30) gB1 (23) gB4 (7) mixed (10)	Blood Urine	1996 (33)
	Germany	99 HIV infected patients (29 CMV retinitis, 70 asymptomatic)	CMV retinitis gB2 (41) gB1 (17) mixed (17) gB3 (14) gB4 (10)	Asymptomatic gB1 (37) gB2 (32) mixed (15) gB4 (9) gB3 (7)	Blood Urine Aqueous humor	1996 (30)
Asia	Japan	54 patients with high ATL (≥50 IU/l)	gB1 (83.3) gB3 (16.7)		Urine	2004 (177)
	Japan	19 patients (5 asymptomtic, 14 symptomic)	Asymptomatic gB1 (60) gB3 (40)	Symptomatic gB1 (100)	-	2002 (181)
	India	26 CMV infected patients	gB2 (53.8) gB3 (46.1)		-	2002 (182)
	Japan	33 Immunocompetent children	gB1 (72.73) gB3 (18.18) gB2 (3.03) mixed (6.06)		-	2000 (178)
	Japan	27 Pediatric BMT	gB2 (75) gB1 (20) gB3 (5)		Serum	1997 (40)

Area	Country	Study population	gB genotype (%)		Specimen	Year (Reference)
Asia	Hong Kong	22 BMT w CMV disease 11 BMT w/o CMV disease 14 renal Tx w CMV disease 13 renal Tx w/o CMV disease 13 asymptomatic premature	BMT w CMV disease gB2 (54) gB1 (14) gB3 (14) gB4 (9) mixed (9)	BMT w/o CMV disease gB1 (46) gB3 (27) gB2 (18) mixed 9)	PBL BAL biopsy	1997 (34)
			Renal Tx w CMV disease gB1 (50) gB3 (21) gB4 (14) gB2 (7) mixed (7)	Renal Tx w/o CMV disease gB1 (69) gB3 (23) gB4 (8)		
			Asymptomatic premature gB1 (77) mixed (23)			

[a] BMT : Bone marrow transplant patients

[b] Tx : Transplant

[c] w : with

[d] w/o : with out

[e] GBS : Guillain-Barre Syndrome

CHAPTER IV

MATERIALS AND METHODS

Part I. Viruses

1. Standard HCMV

HCMV strain AD169 was kindly provided by Mrs. Sukjai Pholampaisathit of the Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand.

2. Clinical specimens

All types of clinical specimens, such as plasma, WBC, tissue, CSF, sigmoid, duodenum, BAL, throat washing, biopsy bronchoscope, lung biopsy and tissue biopsy with HCMV-DNA positive detection by either in-house PCR or PCR amplicor, were recruited in this study. A total of 161 specimens from 128 patients were obtained. They were from Virology Unit, King Chulalongkorn Memorial Hospital during the year 2000 to 2004.

Part II. Preparation of viral DNAs

1. Preparation of viral DNAs by QIAamp®DNA Mini Kit

DNA was extracted from clinical specimens by QIAamp®DNA Mini Kit. The procedure of DNA extraction was followed the recommendation of the company. In brief, 400 µl of clinical specimen was added with 40 µl proteinase K, 400 µl buffer AL and mixed by pulse-vortexing for 15 seconds. After lysis for 10 minutes at 56°C to reach DNA yield, DNA was precipitated by 400 µl absolute ethanol and mixed again by pulse-vortexing for 15 seconds. To remove drops from the inside of the lid, the tube was spined at 8,000 rpm for 1 minute. The mixture was transferred to QIAamp spin column and centrifuged at 8,000 rpm for 1 minute. QIAamp spin column was then washed with 500 µl buffer AW1 and 500 µl buffer AW2 at 8,000 rpm for 1 minute and 3 minutes, respectively. Finally, elution with 20 µl TE buffer (Appendix IV) as performed at 8,000 rpm for 1 minute once and another elution was done after incubating at room

temperature for 5 minutes. The eluate containing viral DNA was stored at -20°C until used.

The standard HCMV strain AD169 was grown on human fibroblast (HF) cells. The infected cells were extracted by using QIAamp®DNA Mini Kit as manufacturer's description. Normal HF-DNA was also prepared and used as negative control.

2. Quantitation of the extracted DNA

The amount of DNA was determined by using an ultraviolet spectrophotometer (SmartSpec^{TM} 3000, Bio-Rad, U.S.A.). Concentration of DNA in the eluate was measured by absorbance at 260 nm. An OD of 1 corresponds to approximately 50 µg/ml for ds-DNA. The ratio of absorbance at 260 nm to absorbance at 280 nm (A_{260}/A_{280}) provides an estimation of purity of nucleic acid, which is A_{260}/A_{280} ratio between 1.7 and 1.9. If there is contamination with protein and other contaminants, the A_{260}/A_{280} ratio will be significantly less than these values.

Part III. HCMV gB genotyping

1. Amplification of HCMV gB gene by nested PCR

PCR typing of HCMV was applied from previously described by Chou, S. and Dennison, K.M. 1991 (18). The Oligonucleotide primers used for PCR amplification were chosen to cover a region of high sequence variability in the HCMV gB gene (nt 1319-nt1604), as shown in Table 5. The first and second rounds of amplification were performed in total volume of 50 µl using 5 µl DNA extract and 2 µl of first PCR product, respectively. PCR mixture composed of 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM dNTPs, 1 unit of Taq polymerase and 20 µM of each primers. This underwent initial pre-incubation at 95°C for 3 minutes; 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute (60°C at 2nd round) and extension at 72°C for 2 minutes; followed by a final extension at 72°C for 5 minutes. After that, 10 µl of the amplified product (293-296 bp, size varies by strain) was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The AD169-DNA was used as positive control, HF DNA and distilled water were used as the negative control.

Table 5. gB Primers Sequences (18).

Round	Primer Sequences		PCR
	Forword	Reverse	Product
1st	gB1043:	gB1724:	700 bp
	TCTGGGAAGCCTCGGAACG	GAGTAGCAGCGTCCTGGCGA	
2nd	gB1319:	gB1604:	293-296 bp
	TGGAACTGGAACGTTTGGC	GAAACGCGCGGCAATCGG	

2. Restriction enzyme digestion

Amplified gB products were cut with restriction enzyme *Hinf* I and *Rsa* I. Approximately 10 µl of nested PCR product were digested at 37°C for 1 hour, using 1 unit of the restriction enzyme. Digesting DNA fragments were separated by electrophoresis at 80 volts in a 7% (vol/vol) polyacrylamide gel and were detected by staining with ethidium bromide.

3. Interpretation of gB genotypes by nested PCR-RFLP

The target amplified by inner primer (gB1319/gB1604) was digested with either restriction enzyme *Hinf*I or *Rsa*I. The results from this analysis could divide into 4 gB genotypes distinguishing by their different patterns of fragment lengths as previously described (18). HCMV gB type1 and 2 were separated from type3 and 4 by *Rsa*I restriction site. In addition, digestion with *Rsa*I may differentiate gB1 from gB2 and gB3 from gB4 (Table 6). If the results show different patterns from those, untype will be named.

Table 6. Restriction fragment length of HCMV gB gene product in each genotype.

HCMV gB genotype	Length of restriction fragments (bp)	
	*Rsa*I	*Hinf*I
1	239, 66	202, 67, 36
2	239, 63	202, 100
3	195, 63, 41	202, 97
4	195, 66, 44	202, 69, 36

Part IV. HCMV gB cloning

1. Preparation of competent cells

　　Escherichia coli strain DH5α was used to perform competent cell. *E. coli* was kindly provided by Assistant Professor Chintana Chirathaworn, Ph.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Briefly, *E. coli* was subcultured on Luria-Bertani (LB) agar plate (Appendix IV) at 37 ℃ overnight. The isolated colony was picked up to culture in 5 ml of LB broth (Appendix IV) and incubated at 37 ℃ overnight. To expand cell culture, 500 μl of that cell suspension was added into 50 ml of LB broth and incubated in shaker incubator at 37 ℃ for 2-3 hours. The amount of cell, was measured by absorbance 600 nm, was adjusted in the range between 0.4 and 0.5. After that, cell suspension was placed on ice for 30 minutes and centrifuged at 5,000 rpm, 4 ℃ for 8 minutes. After removing of supernatant, the pellet was resuspended with 10 ml of 15 % glycerol in mM $CaCl_2$ (Ca/Glycerol solution, Appendix IV), and placed on ice for 30 minutes. Finally, cell suspension was performed by adding cold Ca/Glycerol solution after centrifugation at 5,000 rpm, 4 ℃ for 8 minutes. Suspended cell was aliquoted and stored at -70 ℃ until used.

2. HCMV gB cloning

　　gB cloning was done in clinical specimen with mixed gB genotypes to define the exact HCMV gB combination in each HCMV gB mixed sample.

gB purified PCR product was cloned following pGEM®-T Vector Systems technical manual (Promega, USA). In brief, transformants were transformed to competent cells (*Escherichia coli* strain DH5α) for transformation. Transformed bacterias, had vector containing HCMV gB gene, were selected from LB plate with ampicillin/IPTG/X-Gal. White colony was picked and grown in LB broth and incubated for 16-24 hours at 37°C with shaking. After that, plasmids of transformant bacterias were extracted by QIAprep® Miniprep Kit (QIAgen, Germany). The suspended cells were centrifuged at 4,000 rpm for 10 minutes. The pellet was resuspended with digestion buffer (250 μl buffer P1, 250 μl buffer P2 and buffer N3), mixed gently by inverting the tube, and centrifuged at 13,000 for 1 minute. The supernatant was transferred to the QIAprep spin column and centrifuged at 13,000 for 1 minute. 500 μl buffer PB was added to wash the DNA. Twice washing were performed by adding 350 buffer PE. Finally, DNA elution was performed by incubation with 20 μl buffer EB for 5 minutes and centrifuged at 13,000 for 1 minute. The eluted DNA was kept at -20°C. This DNA was further used for gB amplification.

Part V. HCMV gB sequencing

Specimens which consisted of each unique gB genotype and the independent gB clones were sampling for sequencing. They were analyzed for the variation of HCMV gB sequences.

1. Purification of PCR product

HCMV gB amplicons were purified by QIAquick® PCR Purification Kit (Qiagen, Germany). In brief, adding 200 μl of buffer PB was mixed with a volume of 40 μl of PCR product to bind DNA. The mixture was applied to the QIAquick column and centrifuged at 13,000 rpm for 1 minute. Twice washing DNA was done by adding 350 μl buffer PE and centrifuged at 13,000 rpm for 1 minute after discarding of the supernatant. After that, the QIAquick column was placed to 1.5 microcentrifuge tube. Finally, DNA elution was performed by incubation with 20 μl buffer EB for 5 minutes and centrifuged at 13,000 for 1 minute. The eluted DNA was kept at -20°C until used.

2. Sequencing of purified DNA

The purified PCR products were sequenced following BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol (Applied Biosystems, USA). Briefly, the amplification of purified PCR product was performed in a total volume of 10 µl. This underwent 25 cycles of denaturation at 96°C for 30 seconds, annealing at 50°C for 10 minutes and extension at 60°C for 4 minutes. The extension product was precipitated with 2.5 µl of 125 mM EDTA and 60 µl of absolute ethanol. Then, centrifugation at 13,000 rpm for 30 minutes after incubating at 4°C for 15 minutes was done. The supernatant was removed from the microcentrifuge tube and the DNA was washed by using 60 µl of 70% ethanol for 10 minutes at 13,000 rpm. Finally, the supernatant was discarded and dried at 95°C for 2 minutes. DNA sequences were analyzed by DNA sequencing using an ABI Prism® 310 Genetic Analyzer. Sequences were aligned by use of the software ClustalX.

Part VI. Study of HCMV UL97 mutation by sequencing

Viral genomic DNA obtained from clinical specimens were selected from patients who had received GCV prophylaxis for more than 3 months. There were only 10 samples from 4 patients, which reached this criterion.

1. PCR amplification of the HCMV *UL97*

HCMV DNA representing codon 428 to 628 of UL97 was amplified by PCR. The oligonucleotide primers, previously published (18), were chosen. *UL97* coding sequence was determined by using of 5' ATCGACAGCTACCGACGTGCC 3' as forward primer and 5' GCCATGCTCGCCCAGGAGACAGG 3' as reverse primer. The amplification was performed in a total volume of 50 µl. This reaction mixture composed of 10 µl DNA extract and 40 µl PCR mixture consisting of 50 mM KCl, 10mM Tris pH8.3, 1.5 mM $MgCl_2$, 10 mM dNTPs, 1U of Taq polymerase in buffer containing 10% DMSO and 20 µM of each primer. PCR reaction underwent 35 cycles of denaturation at 94.5°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes.

2. HCMV *UL97* cloning

    UL97 cloning method was followed the described above in Part IV HCMV gB cloning.

3. Sequencing of HCMV *UL97*

    Purification of *UL97* PCR product and sequencing methods were similar to those described above in Part V HCMV gB sequencing.

Part VII. Statistical analysis

    Most data were presented as descriptive statistic such as mean, standard deviation, median, percentage, frequency etc. ANOVA performed by using SPSS software (version11.0; SPSS) was run for analysing statistically significant difference ($p < 0.5$).

Part VII. Phylogenetic analysis

    The clinical samples and sequences of the reference strains gB1 through gB4 (18) were compared by used of ClustalX and TreeView program for multiple alignment and to generate the phylogenetic trees, respectively. Those references are M60927 (gB1), AD169 (gB2), M60933 (gB3) and M60924 (gB4), derived from GenBank (18).

CHAPTER V

RESULTS

PART I   HCMV gB genotyping

1. Specificity and sensitivity of HCMV gB nested PCR

The specificity of oligonucleotide primers using in gB gene amplification was not shown in the previous report (18). In this study, HSV1 (KOS), HSV2 (Baylor186), and EBV DNA were included to analyze the specificity. These oligonucleotide primers were only specific for HCMV DNA, since they could not amplify the other Herpesvirus DNA (Figure 10A).

For sensitivity, serial 10-fold concentrations of standard HCMV AD169 DNA were amplified by using the gB nested PCR assay. At least 1 fg of HCMV DNA could be detected, as shown in Figure 10B.

2. Detection of HCMV gB gene in clinical specimens

A total of 161 clinical specimens from 128 patients were obtained from Virology Unit, King Chulalongkorn Memorial Hospital during January 2000 to December 2004.

Clinical specimens were 110 plasma, 33 WBC, 5 CSF, 4 BAL, 2 throat-washing, 1 tissue, 1 sigmoid, 1 duodenum, 1 ETT secretion, 1 urine, 1 liver biopsy and 1 lung biopsy. All of them were re-examined by nested PCR against gB. The numbers of positive results in each type of clinical sample were shown in Table 7. 113 out of 161 (70.19 %) samples were successfully amplified. They were from 96 patients including of 48 females and 48 males.

A)

B)
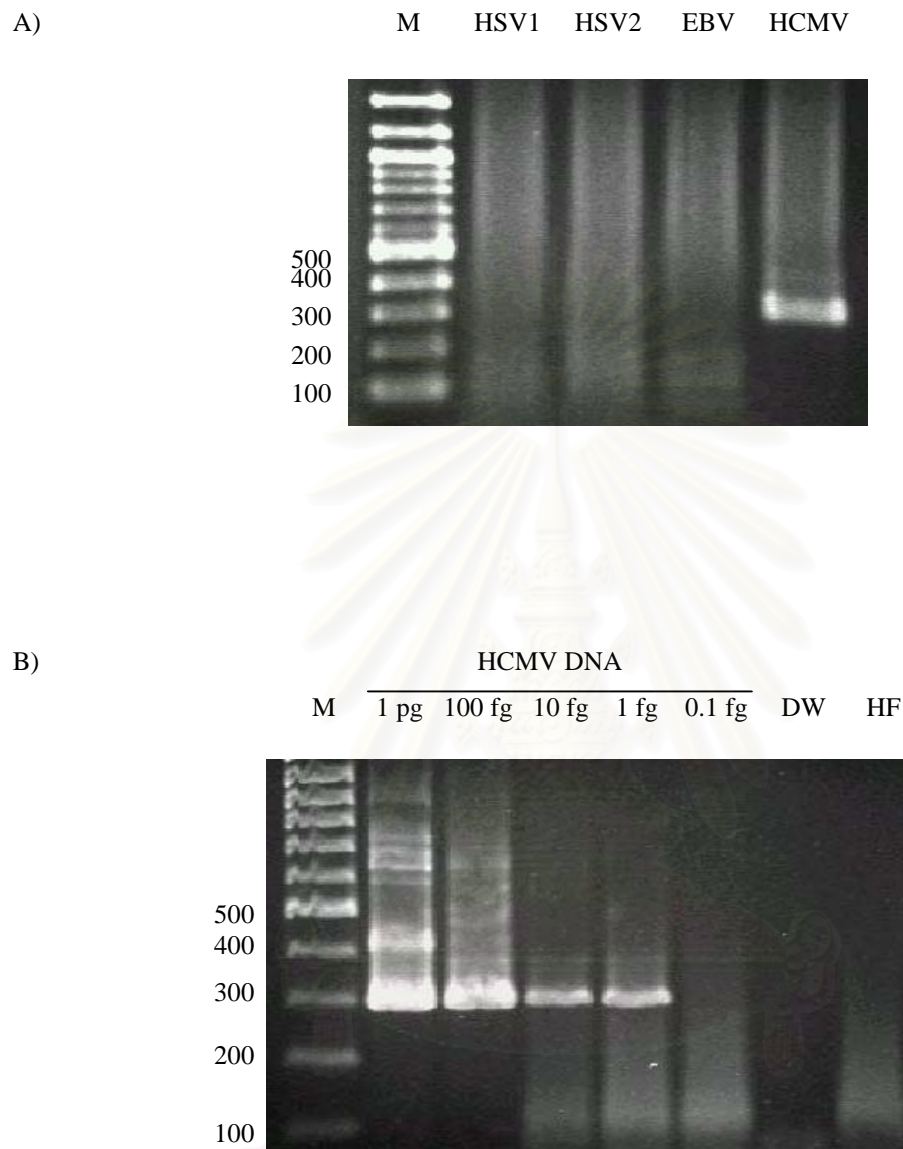
Figure 10. The specificity and sensitivity of the nested PCR assay. A) Specificity of oligonucleotide primers among HCMV AD169 DNA and the other Herpesvirus DNA. B) Ten-fold concentration of 0.1 fg to 1 pg of the HCMV gB gene were amplified by the nested PCR. Lane M, showed the DNA marker (bp). Lane DW and lane HF showed the negative control.

Table 7. The number of positive HCMV gB gene fragment amplification in each type of clinical samples.

Type of sample	Number of samples	Number of gB positive
Plasma	110	87
WBC	33	14
CSF	5	4
Sigmoid	1	1
Duodenum	1	1
BAL	4	2
ETT secretion	1	0
Throat washing	2	1
Urine	1	0
Lung biopsy	1	1
Liver biopsy	1	1
Tissue	1	1
Total	**161**	**113 (70.19 %)**

3. HCMV gB genotyping by RFLP

At least 4 gB genotypes of HCMV are determined by RFLP using restriction enzyme, *Hin*f I and *Rsa* I (18). Four restriction patterns of HCMV gB1 to 4 genotypes were shown in Figure 11. The patterns other than these 4 patterns were identified as untype (UT). In addition, mixed gB genotypes were possibly found. Figure 12 demonstrated the patterns of mixed gB genotypes. Mixed infection of gB1 and3 or gB2 and 4 or more than 2 gB genotypes could not be identified since the patterns were similar. Therefore, we identified as mixed unclassified types (MUT).

HCMV gB genotyping was successful done in 113 specimens of 96 patients. The infection of mixed genotypes was the most frequently (34.51 %), following by gB1 (32.74 %), gB3 (15.04 %), gB2 (10.62 %), and untype (7.08 %). Surprisingly, none of gB4 was demonstrated, as shown in Table 8. However, all together, monotyping of gB genotype was mostly found in clinical samples (65.49 %, 74/113). Of those 8 untyped samples, they were divided into 2 patterns (UT1 and UT2), as shown in Figure 13. Two of them were UT1 and the remainders were UT2. Mixed infection was dominantly found in plasma specimens (36.78 %, 32/87), as shown in Table 8. More than 5 expected patterns of mixed gB genotype (Figure 12) were found, including gB1+2+UT, gB2+3+UT, and gB3+4+UT, as shown in Figure 14. Table 9 demonstrated the distribution of each pattern of mixed gB genotypes in various specimens. Among 8 patterns of gB mixed infection, both combination of gB2 and gB3, and MUT were the most commonly found (30.77 %).
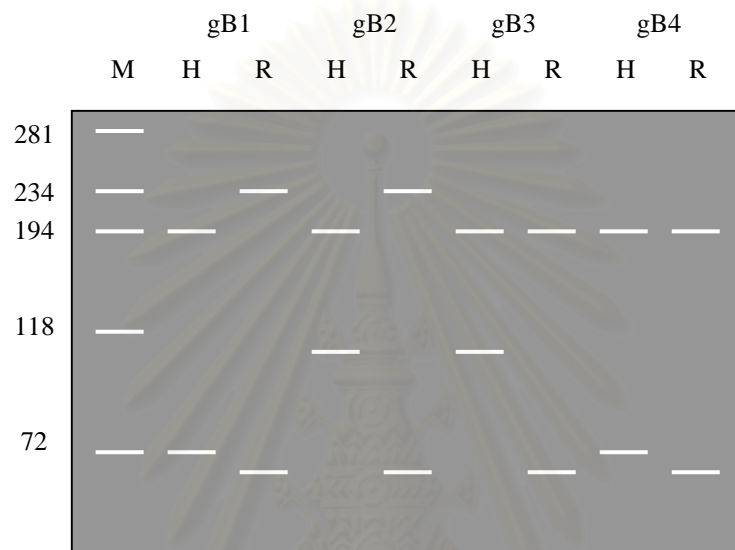
Figure 11. Diagram of restriction digestion pattern of HCMV gB genotype 1 to 4 after Nested PCR assays. M, DNA molecular sizes marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest.
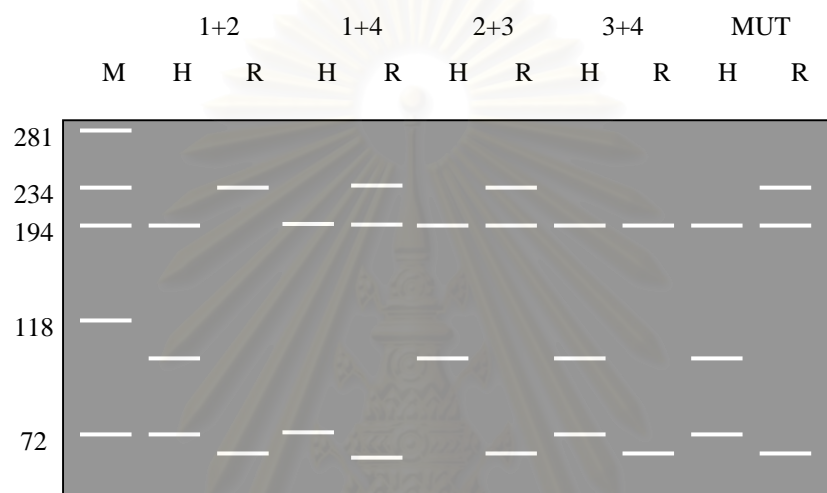
Figure 12. Diagram of restriction digestion pattern of expected HCMV mixed gB genotypes. M, DNA molecular sizes marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest; MUT, mixed unclassified types.

Table 8. Frequency distribution of gB genotype in various specimens.

Type of specimens	gB genotype (%)					
	gB1	gB2	gB3	gB4	Untype	Mixed
Plasma	24 (27.59)	10 (11.49)	15 (17.24)	0 (0)	6 (6.9)	32 (36.78)
WBC	6 (43)	0 (0)	2 (14)	0 (0)	2 (14)	4 (29)
CSF	3 (75)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)
Sigmoid	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Duodenum	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
BAL	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	1(50)
Throat washing	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Lung biopsy	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Liver biopsy	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Tissue	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (113)	**37 (32.74)**	**12 (10.62)**	**17 (15.04)**	**0 (0)**	**8 (7.08)**	**39 (34.51)**

Table 9. Frequency distribution of each mixed gB genotypes in various specimens.

Type of specimens	gB combination							
	1+2	1+4	2+3	3+4	1+2+UT	2+3+UT	3+4+UT	MUT
Plasma	6	2	9	4	1	1	0	9
WBC	0	0	1	0	0	0	1	2
CSF	0	0	1	0	0	0	0	0
BAL	0	0	0	0	0	0	0	1
Liver biopsy	0	0	1	0	0	0	0	0
Total	**6**	**2**	**12**	**4**	**1**	**1**	**1**	**12**
(%)	**15.38**	**5.13**	**30.77**	**10.26**	**2.56**	**2.56**	**2.56**	**30.77**
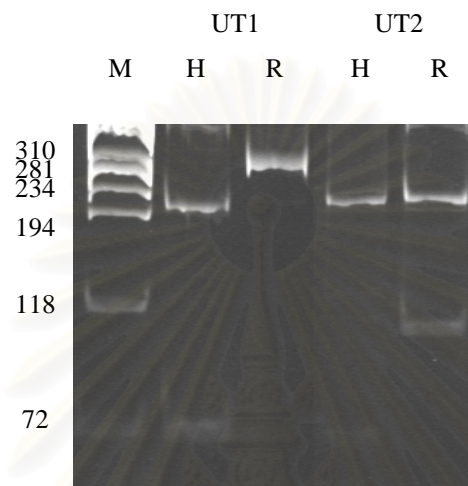
Figure 13. Restriction digestion pattern of the untyped gB genotypes (UT). M, DNA molecular sizes marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest.
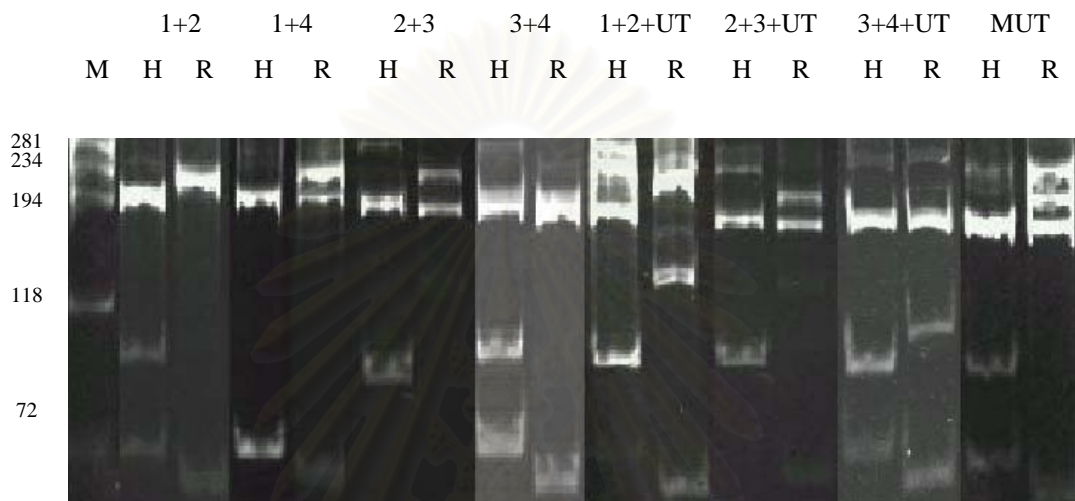
Figure 14. Restriction digestion pattern of the mixture of HCMV gB genotypes including of the expected patterns. M, DNA molecular sizes marker (φx174 *Hae* III digest, bp); H, *Hin*f I digest; R, *Rsa* I digest; UT, untyped genotype.

Five out of 96 patients, who had different or same type of samples collecting at different times, were demonstrated distinct pattern of gB genotype (Table 10). Although, some samples of those patients were gB monotype, either gB2 or gB3, they were defined as mixed gB infection. According to Table 10, although each sample came from the same patient collecting at different times, increasing of different HCMV gB genotype infection was observed especially in plasma samples (No. 27, 28, 39). Two patients (No. 20 and 81) showed different gB genotype in different type of samples. However, there was common type found among those samples (No. 20, gB2; No. 81, gB3).

The distribution of HCMV gB genotypes among 96 patients was shown in Table 10. The majority of patients (37.50 %) were found more than one gB genotype. Those 60 patients had a unique gB genotype including untyped gB. In addition, those 5 patients (3 gB1[a] and gB3[c] patients, Table 10) who had more than one sample, either collecting at the same time or different time, showed no distinct gB genotype. Mixed infection of gB genotypes were defined as the presence of more than one gB genotype either in different samples from the same patient or in a single sample (Table 10-12). There were 31 patients shown mixed gB genotypes following restriction enzyme assay (Table 12). Combination of gB2 and 3, and MUT found to be the most common pattern in patients with mixed infection (32.26 %), following by gB1+2 (12.90 %) and gB3+4 (9.68 %) infections. Within gB3+4 patients, one had 2 clinical samples collecting at different time (Table 12).

Table 10. HCMV gB genotypes combination represent more than one pattern in the same person.

Number	Code	Date	Sample	gB genotype
20	6F	14-8-03	Plasma	1+2
	2E	29-8-03	Throat washing	2
	3E	8-9-03	BAL	2
27	5F	15-7-02	Plasma	3
	9I	12-13-02	Plasma	2+3
28	2I	21-1-01	Plasma	1+4
	8F	21-2-04	Plasma	1+2
	3I	23-3-04	Plasma	MUT
39	6B-2	24-4-03	Plasma	2
	4H	9-7-03	Plasma	1+2+untype
81	10H-2	27-8-04	Plasma	3
	2I-2	3-9-04	Liver biopsy	2+3

Table 11. HCMV gB genotypic distribution of 108 clinical samples from 96 patients.

gB genotype infection	Number of specimens	Number of patients (%)
gB1	37	33 (34.38) [a]
gB2 [b]	9	7 (7.29)
gB3	15	13 (13.54) [c]
gB4	0	0 (0)
Untype	8	7 (7.29)
Mixed	39	36 (37.50)

a   Three patients had different type of samples collecting at the same time.
     (1 patient: CSF, sigmoid and duodenum; 2 patients: plasma and WBC)
b   3 samples of gB2 and 2 samples of gB3 were excluded because these patients
     were already defined as mixed infection.
c   One patient had 2 different types of samples collecting at the same time
     (plasma and WBC) and 1 patient had 2 plasma samples collecting at different times.

Table 12. HCMV gB genotypes combination represent one pattern in the same person.

Combination of gB genotype	Number of clinical samples (%)	Number of patients (%)
1+2	4	4 (12.90)
1+4	1	1 (3.22)
2+3	10	10 (32.26)
3+4	4	3* (9.68)
1+2+untype	1	1 (3.22)
2+3+untype	1	1 (3.22)
3+4+untype	1	1 (3.22)
MUT	10	10 (32.26)
Total	**32**	**31**

* One of 3 patients had 2 clinical samples (plasma) collecting different time.

The frequency distribution of gB genotypes between male and female patients was compared, as shown in Figure 15. Discrete data among gender was shown in Table 13. The gB2, gB3 and mixed gB genotypes were comparable distributed between sex. In contrast, the gB1 was found less frequently in female (27.08 %) and untyped gB genotype was found less frequently in male (4.17 %)

The HCMV gB genotype was determined in immunocompromised patients including of known 31 HIV infected patients and known 13 transplant recipients. The distribution of the gB genotypes in HIV infected patients and transplant recipients was shown in Figure 16 and discrete data was shown in Table 14. Genotype gB1 (61.54 %) was the predominantly found in 13 transplant recipients, whereas mixed gB genotypes was the most frequent in 31 HIV infected patients (35.48 %). Interestingly, gB2 was found only in transplant recipients, while untype was detected only in HIV-infected patients.

HCMV load was compared in samples with different HCMV gB genotypes. These HCMV loads derived from 59 clinical specimens with PCR amplicor positive. The median value of HCMV load for each genotype was followed: gB1, 45,000 copies/ml (range 791->100,000 copies/ml); gB2, 4,215 copies/ml (range, 2,220->100,000 copies/ml); gB3, 14,600 copies/ml (range, 1,210->100,000 copies/ml); and untype, 24,500 copies/ml (range, 7,350-88,700 copies/ml). All together, the median value of peak virus load for single gB genotype infection was 16,850 copies/ml (range, 791->100,000 copies/ml) and mixed infection was 16,350 copies/ml (range, 561->100,000 copies/ml) as shown in Figure 17. No association between gB genotype and level of HCMV viral load was detected ($p = 0.712$).

Frequency distribution of gB genotype between female and male
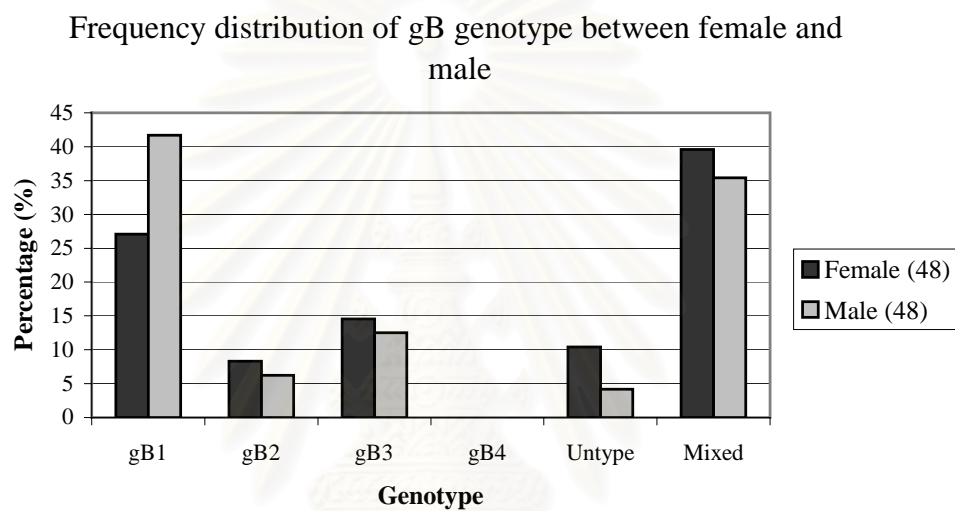
Figure 15. Frequency distribution of gB genotype between female and male. The population included of 48 females and 48 males.

Table 13. Discrete data of gB genotype between female and male. The population included of 48 females and 48 males.

gB genotype	No. Female (%)	No. Male (%)
1	13 (27.08)	20 (41.67)
2	4 (8.33)	3 (6.25)
3	7 (14.58)	6 (12.5)
4	0 (0)	0 (0)
Untype	5 (10.42)	2 (4.17)
Mixed	19 (39.50)	17 (35.42)
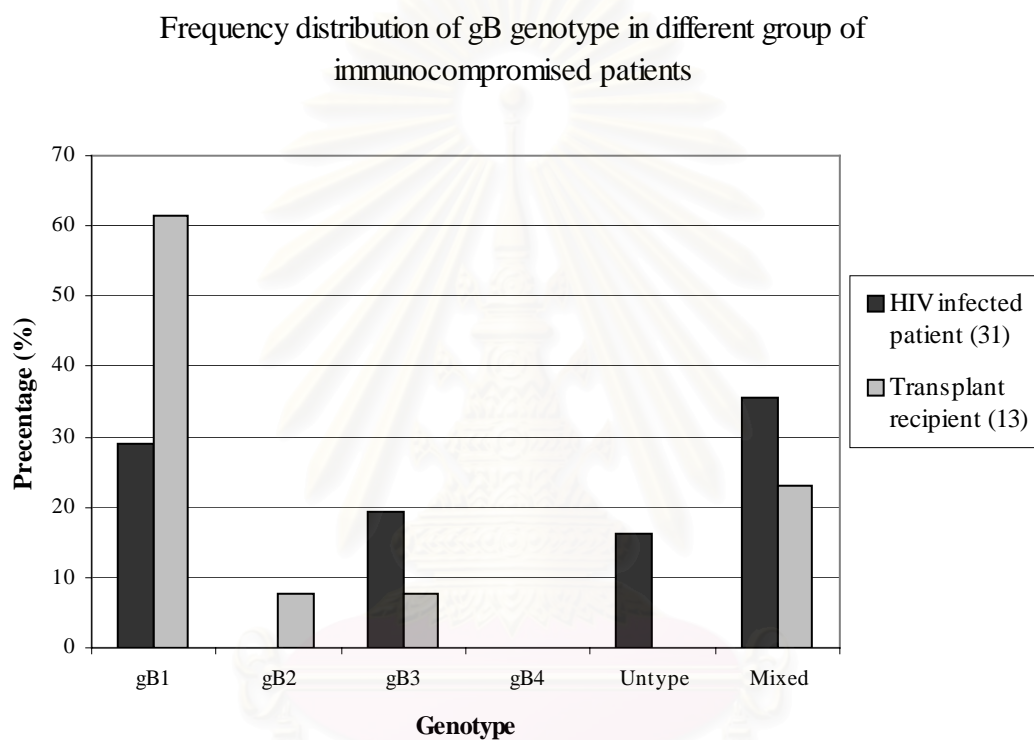
Figure 16. Frequency distribution of gB genotype in different groups of immunocompromised patients. The population included of 31 HIV infected patients and 13 Transplant recipients.

Table 14. Discrete data of gB genotype in different groups of immunocompromised patients. The population included of 31 HIV infected patients and 13 Transplant recipients.

gB genotype	No. HIV infected patients (%)	No. Transplant patients (%)
1	9 (29.03)	8 (61.54)
2	0 (0)	1 (7.69)
3	6 (19.35)	1 (7.69)
4	0 (0)	0 (0)
Untype	5 (16.13)	0 (0)
Mixed	11 (35.48)	3 (23.08)
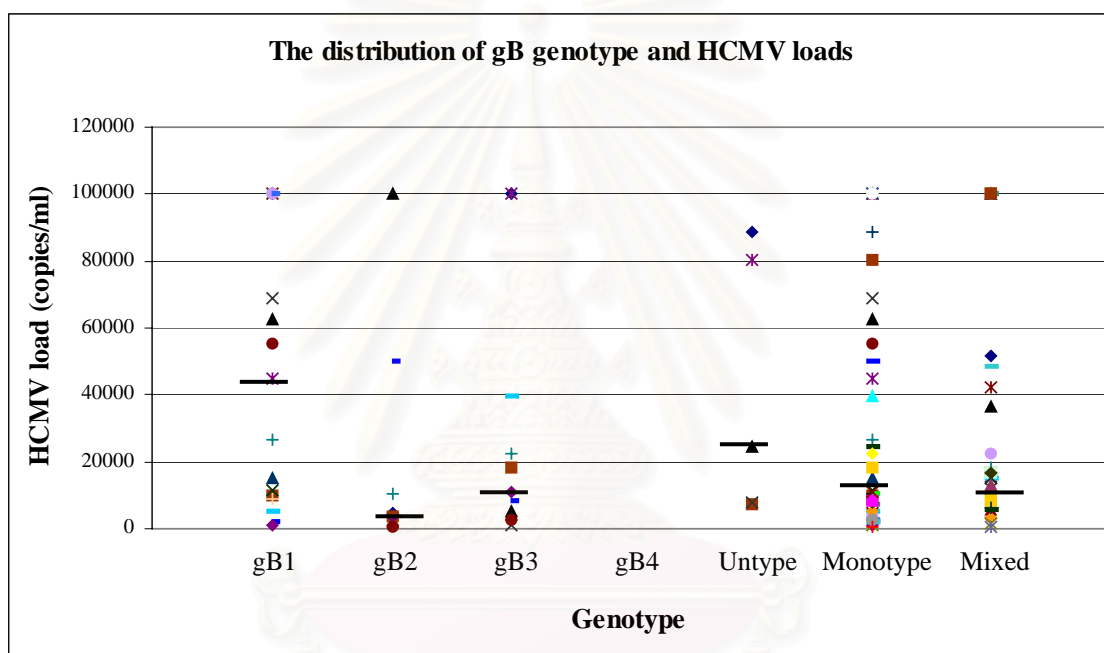
Figure 17. HCMV gB genotype and HCMV load in plasma samples. Median of each group was marked (——).

4. HCMV gB cloning

Cloning method was used to determine gB genotype in RFLP defining mixed gB genotypes. Three clinical samples composing of 1 gB3+gB4 sample and 2 MUT were selected. A total number of successive transforming clones and gB genotyping were showed in Table 15.

Surprisingly, gB4 did not be found in 2F clinical specimen (gB3+gB4 combination). Despite the only gB3 and untype were defined, the union of both restriction patterns could be interpreted into gB3 and gB4 combination, as shown in Figure 18. The untype restriction pattern of 2F successive transforming clones related to UT1 patterns. 6H sample (MUT) could be defined at least 2 gB genotypes, for instance gB1 and gB3 (Figure19). While, 1I sample was shown to combine at least 3 gB genotypes (gB1, gB2 and gB3), as shown in Figure 20.

Besides 3 samples of mixed infection, gB2 clinical sample (5B-2) was also cloned to study intragenotypic variation in the same person.

Table 15. The cloning of mixed gB genotypes and unique gB genotype of successive transformants.

Code	Combination of gB genotype	No. of successive transforming	gB genotype (No. of clones)
2F	3+4	16	gB3 (8) Untype (8)
6H	MUT	23	gB1 (19) gB3 (4)
1I	MUT	8	gB1 (5) gB2 (1) gB3 (2)
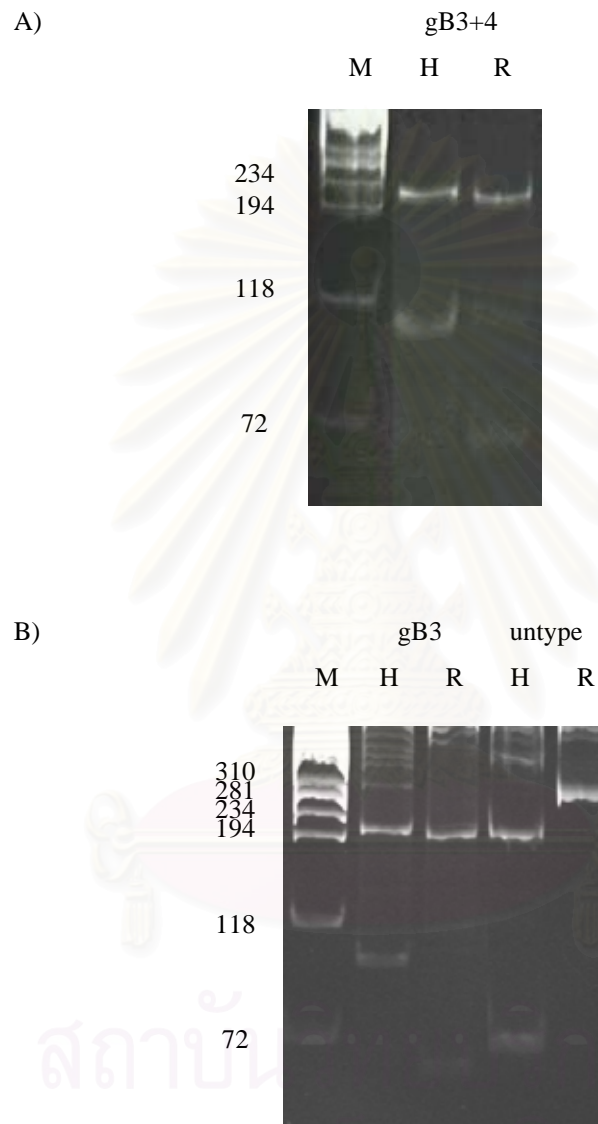
Figure 18. The restriction pattern of 2F clinical specimen. A) The restriction pattern of 2F before gB gene cloning. B) The restriction pattern of 2F independent clones. Lane:M, DNA molecular size marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest.

A)

MUT

M H R

234
194

118

72

B)

gB3 gB1

M H R H R

234
194

118

72
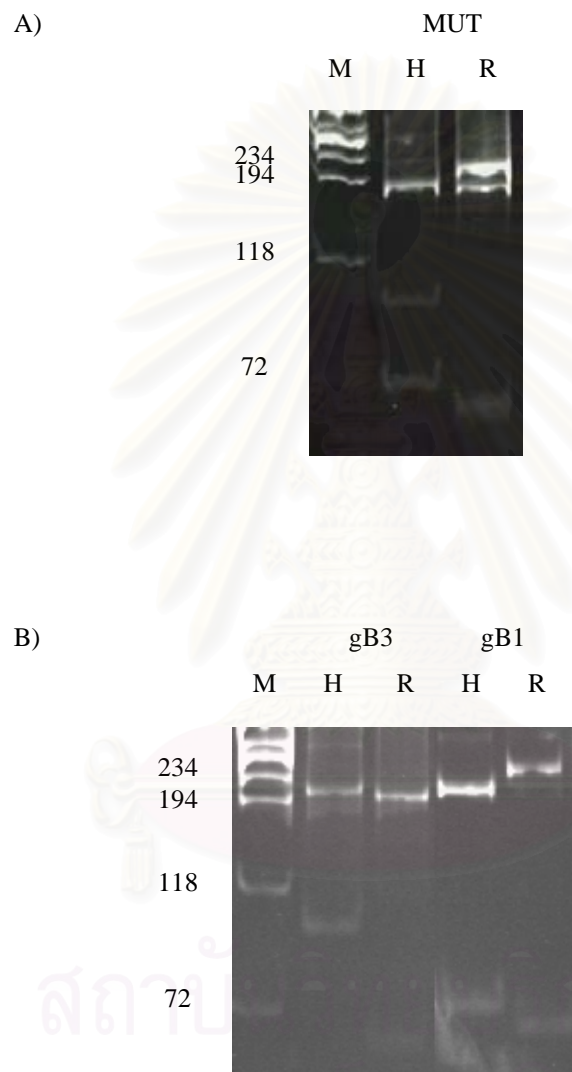
Figure 19. The restriction pattern of 6H clinical specimen. A) The restriction pattern of 6H before gB gene cloning. B) The restriction pattern of 6H independent clones. Lane:M, DNA molecular size marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest.
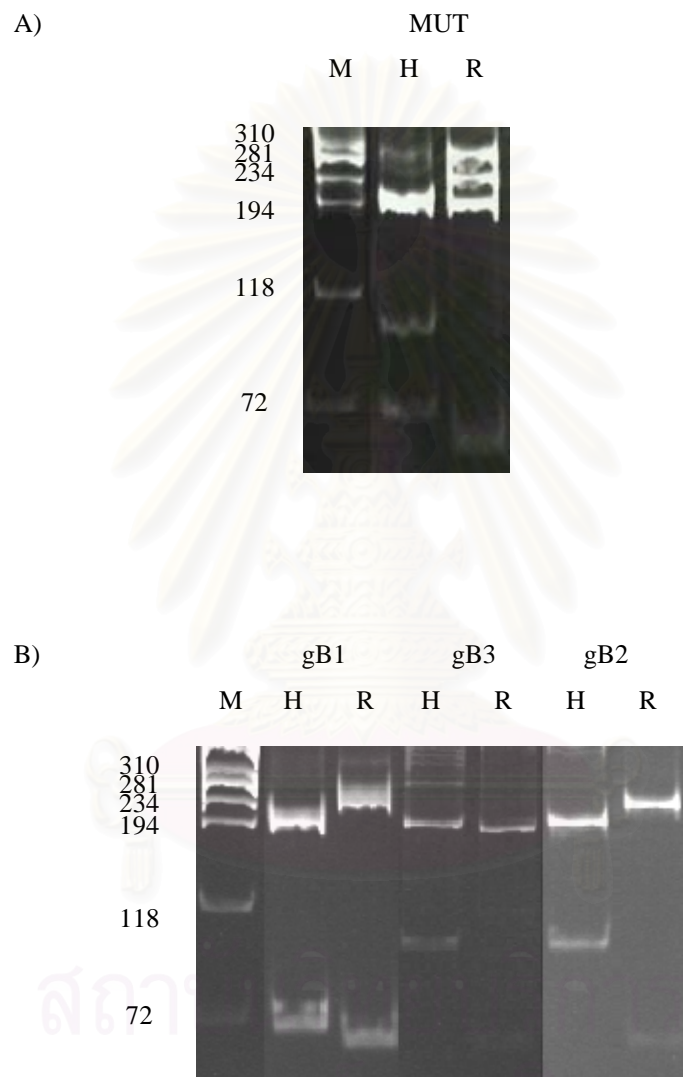
Figure 20. The restriction pattern of 1I clinical specimen. A) The restriction pattern of 1I before gB gene cloning. B) The restriction pattern of 1I independent clones. Lane:M, DNA molecular size marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest.

5. HCMV gB sequencing

Clinical specimens in each gB genotype were sampling for sequencing. 8 gB1, 6 gB2, 7 gB3 and 6 untype samples were done. All of these sequences were restriction mapped for their gB genotype to confirm RFLP analysis by BioEdit program. Surprisingly, the result of restriction map analysis, one of gB1 (5D) sample was defined as gB3 restriction sites. Sequence of RFLP defining gB1 (5D) has the mutation in *Hin*f I cleavage site, resulting in 202, and 97 bp fragments instead of 202, 69 and 36 bp fragments. In addition, there is the mutation in *Rsa* I cleavage site, resulting in 195, 63, and 41 bp fragments instead of 239, and 66 bp fragments (Figure 21).

Although, some sequences were shown controversial, all sequences were aligned and subsequently assigned to 1 of the 4 described gB genotype by ClustalX (1.81) software and program TreeView, respectively. M60927 (gB1), AD169 (gB2), M60933 (gB3), and M60924 (gB4) were used to be reference strains. Phylogenetic tree analysis of HCMV gB genotype was shown in Figure 22. 7 of the 8 RFLP defining gB1 sequences, 2 of the 6 RFLP defining gB2 sequences, and all of the 7 RFLP defining gB3 sequences were clustered with the reference strain of each gB genotype. In contrast, one sample of RFLP defining gB1 (5D) and 5 samples of gB2 (5K-2, 2E, 6B-2, 8A and 1IC12) were clustered with the M60933, gB3. Almost RFLP defining untype sequences were clustered with M60927 (gB1) in spite of one clinical strain (9D), was clustered distinctively branch of the same origin to M60933, gB3 (Figure 22).

In the gB coding region (bases 1336-1569), nucleotide homology of clinical strains within each genotype based on RFLP pattern was shown in Table 16. Table 17 showed nucleotide sequence similarity of both UT1 and UT2 genotypes comparing to 4 gB reference sequences. The gB1 sample (5D) that was analyzed 77.12 % homology, had 96.96 % homology with the reference strain of gB3 (Table 16). Among 3 of 7 gB2 sequences were 100 % similarity to the reference strain. The other 5 strains (8A, 2E, 6B-2, 5K-2 and 1IC12) of gB2 sequences were closed to gB3 (range, 96.96-98.70 %), although the sequencing of restriction site was confirmed to be gB2. However, these 5 samples were defined as gB3 genotypes later in this study. Table 18 showed nucleotide homology between HCMV clinical strains, which analyzed according to gB genotype based on sequence, with the exception of 9D UT1 sequence.

A)

gB1

M   H   R

234
194

118

72

B)
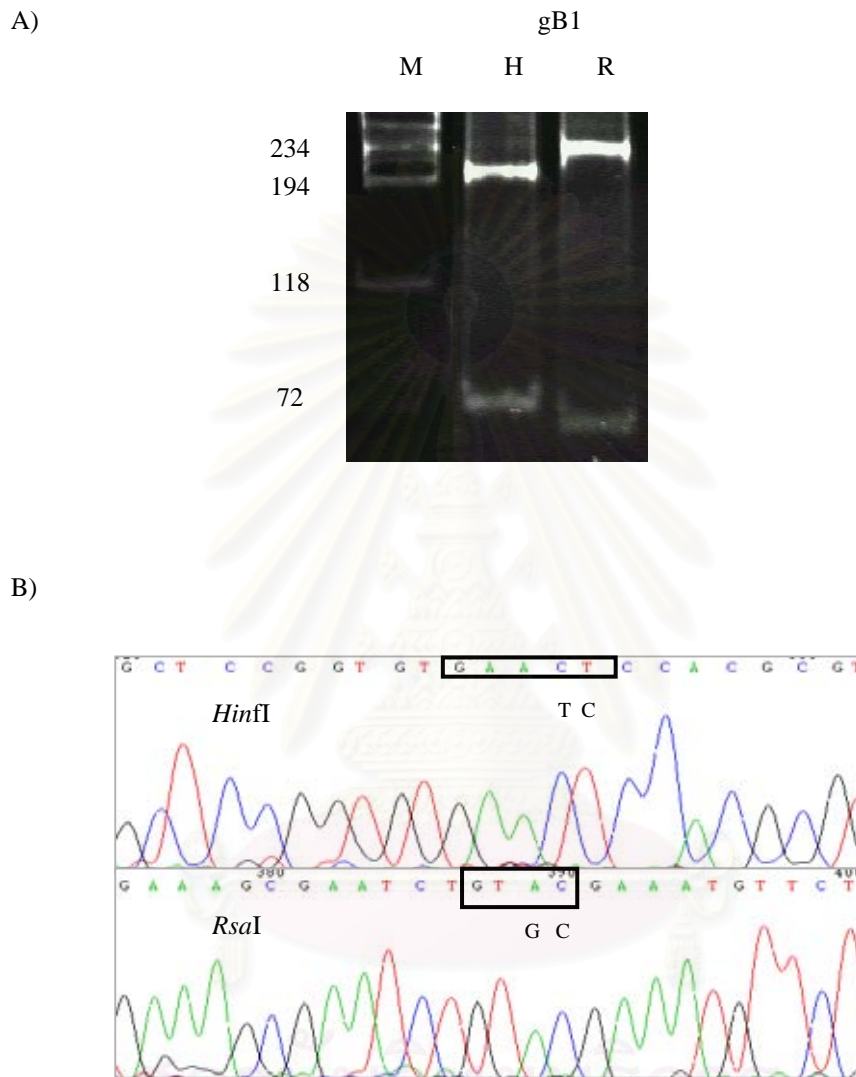
Figure 21. 5D showed 2 distinct of gB genotype interpretation between PCR-RFLP analysis and restriction map of gB sequence by BioEdit program. A) RFLP defining gB1 pattern. Lane:M, DNA molecular size marker (φx174 *Hae*III digest, bp); H, *Hin*fI digest; R, *Rsa*I digest. B) Mutation of gB sequence related to changing of enzyme restriction sites like gB3. The square blankets represent enzyme recognition site. The letters under the blanket showed the substituted nucleotides.
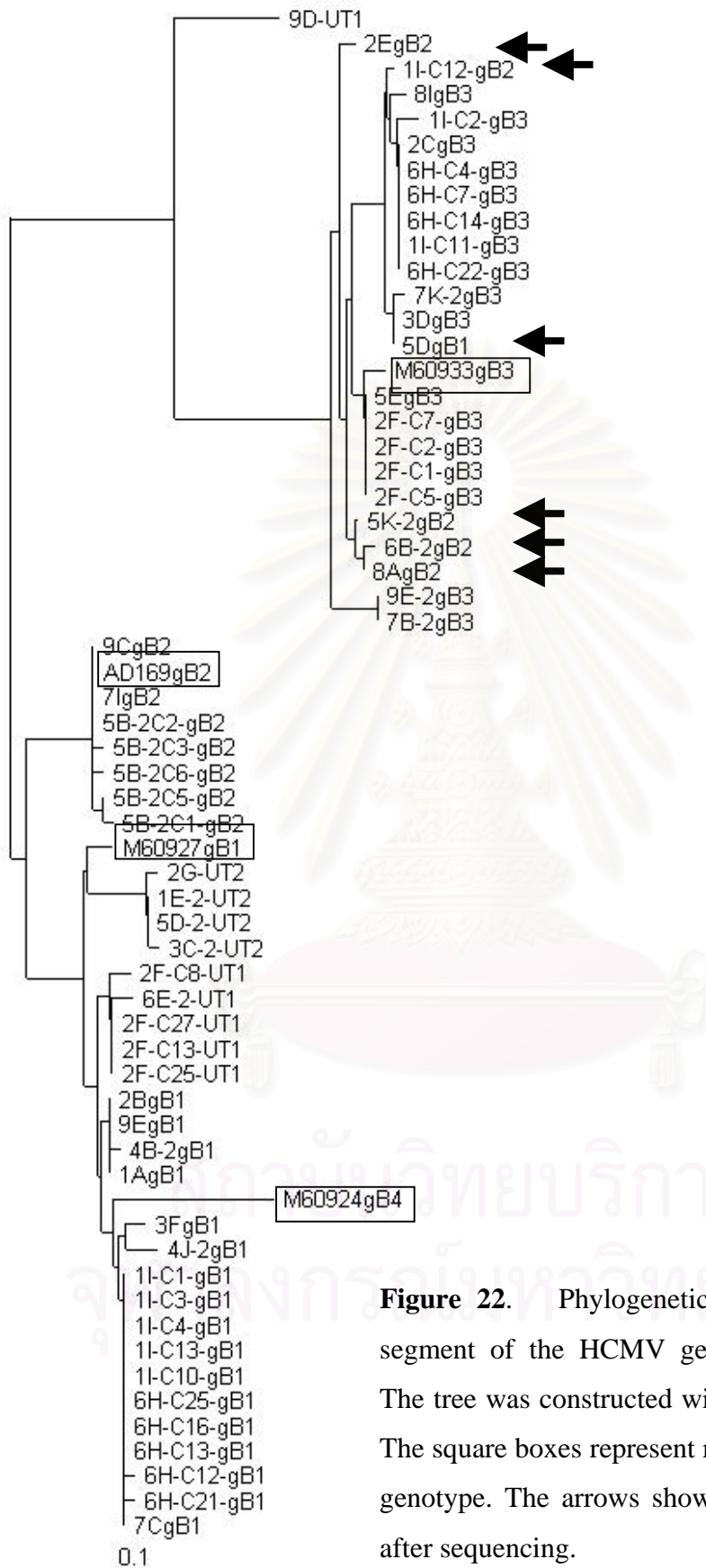
Figure 22. Phylogenetic tree analysis of the gB segment of the HCMV genome (bases 1336-1569). The tree was constructed with the program TreeView. The square boxes represent reference strain of each gB genotype. The arrows showed different gB genotype after sequencing.

Table 16. Nucleotide homology of clinical strains within each HCMV gB genotype.

gB genotype based on RFLP	Code	% Nucleotide homology
gB1	1A	97.88
	2B	98.30
	7C	97.46
	5D	**77.12 (96.96) ***
	9E	97.88
	3F	97.88
	4B-2	97.46
	4J-2	96.61
	1IC1	97.46
	1IC3	97.46
	1IC4	97.46
	1IC10	97.46
	1IC13	97.46
	6HC12	97.03
	6HC13	97.46
	6HC16	97.46
	6HC21	97.03
	6HC25	97.46
gB2	**8A**	**81.12 (97.83) ***
	2E	**81.54 (96.96) ***
	7I	100.00
	6B-2	**80.69 (97.39) ***
	9C	100.00
	5K-2	**79.40 (98.70) ***
	5B-2C1	99.14
	5B-2C2	100.00
	5B-2C3	99.57
	5B-2C5	99.57
	5B-2C6	99.57
	1IC12	**79.83 (97.39) ***
gB3	2C	97.83
	3D	97.39
	5E	99.56
	8I	96.96
	7B-2	96.96
	9E-2	96.96
	7K-2	98.26
	2FC1	99.56
	2FC2	99.56
	2FC5	99.56
	2FC7	99.56
	6HC4	97.83
	6HC7	97.83
	6HC14	97.83
	6HC22	97.83
	1IC2	97.83
	1IC11	97.83

<u>Note</u> The thick letters represent the nucleotide homology of the clinical strains that were clustered as gB3 genotype. * % nucleotide homology when compared to M90633 (gB3). The square boxes were cloned samples.

Table 17. Nucleotide homology of untype clinical strains with 4 gB reference strains.

gB genotype based on RFLP	Code	Nucleotide homology (%)			
		M60927 (gB1)	AD169 (gB2)	M60933 (gB3)	M60924 (gB4)
UT1	9D	85.17	84.12	87.83	80.93
	6E-2	96.19	91.42	77.83	92.37
	2FC8	97.03	92.27	78.69	92.80
	2FC13	97.03	92.27	78.69	92.80
	2FC25	97.03	92.27	78.69	92.80
	2FC27	97.03	92.27	78.69	92.80
UT2	2G	97.46	90.13	78.26	91.52
	3C-2	97.03	90.56	77.83	91.95
	5D-2	97.46	90.13	78.26	92.37
	1E-2	97.46	90.13	78.26	92.37

Note   The square box was cloned samples.

Table 18. Nucleotide homology between HCMV clinical strains.

gB genotype of clinical strains	Nucleotide homology (%)			
	M60927 (gB1)	AD169 (gB2)	M60933 (gB3)	M60924 (gB4)
gB1	**97.48**	92.42	77.78	92.75
gB2	91.64	**99.69**	79.81	87.41
gB3	77.71	79.67	**98.02**	76.68
Untype	**97.08**	91.27	78.36	92.42

To analyze nucleotide sequence variation among gB genotypes within the same person, the independent clones of 3 specimens with mixed infection (2F, 6H and 1I) and 5B-2 independent clones were sampling for gB sequencing. All of 1I independent clones were sequenced. Whereas, 2F successive clones were sequenced only 4 gB3 clones and 4 gB untype clones. Five gB1 clones and 4 gB3 clones of 6H clinical specimen were also sequenced (Table 15).

Nucleotide sequence similarities between each clone were already shown in Table 16 and Table 17. The variation of nucleotide and amino acid sequences of different gB genotype from 2F, 6H, 1I and 5B-2 were showed in Figure 23, Figure 24, Figure 25 and Figure 26, respectively comparing to the reference strains. Intragenotypic variation could be found in 2F UT1, 6H gB1, 1I gB3 and 5B-2 gB2 sequences. While, all of 2F gB1, 6H gB3 and 1I gB1 sequences were identical among themselves. To take note of 1I gB2 sequence (1I-C12), it was included to be gB3, since it closed to M60933 (gB3) with 97.39 % similarity (Table16). All together, nucleotide homologies between clinical strains were analyzed. According to genotyping based on sequencing, sequence similarity on the average was found to be 97.48 % in gB1, 99.69 % in gB2 and 98.02 % in gB3. In addition, all of both UT1 and UT2 sequences were clustered with M60927 (gB1) with the exception of 9D UT1 sequence (Figure 22) and nucleotide homology among them was the most nearly to M60927 with the range of 96.19-97.46 %, as shown in Table17.

Based on predicted peptide sequences, 78 codons (codons 446-523) were analyzed. Of 78 codons, 37 codons showed nucleotide variation affecting amino acid substitution, as shown in Figure 27. Comparing to the reference strain, 7 patterns of variants peptide sequences were shown in 17 gB1 sequences. While, there are 5 patterns and 9 patterns of variants peptide sequences among 7 gB2 and 23 gB3 sequences, respectively. Codon 518 was the most variable position, found in gB1 (Arg to Lys) and gB3 (Lys to Arg). In addition, an Arg to Gln mutation at codon 497 and an Asn to Asp mutation at codon 463 were also frequently found in gB3 and gB1, respectively. Among UT sequences, UT1 could be separated from UT2 by codons 458, 467, 472 and 518, as shown in Figure 27.

A) 2F gB3 sequences

                                                471

M60933 CGT AGA ACC AAG AGA AGT ACG GGC AAT ACG ACC ACC CTG TCA CTT

                                             Ser (S) Leu (L)

2FC1   CGT AGA ACC AAG AGA AGT ACG GGC AAT ACG ACC ACC CTG TC**G** C**C**T

2FC5   CGT AGA ACC AAG AGA AGT ACG GGC AAT ACG ACC ACC CTG TC**G** C**C**T

2FC7   CGT AGA ACC AAG AGA AGT ACG GGC AAT ACG ACC ACC CTG TC**G** C**C**T

2FC2   CGT AGA ACC AAG AGA AGT ACG GGC AAT ACG ACC ACC CTG TC**G** C**C**T

                                             Ser(S) Pro (P)

B) 2F UT1 sequences

                                 513

2FC13 TGG TGT GTG GAT CAA CGG CGC AGC CTA GAG GTC TTC AAG GAA CTC

2FC25 TGG TGT GTG GAT CAA CGG CGC AGC CTA GAG GTC TTC AAG GAA CTC

2FC27 TGG TGT GTG GAT CAA CGG CGC AGC CTA GAG GTC TTC AAG GAA CTC

                                  Ser (R)   Val (V)

2FC8   TGG TGT GTG GAT CAA CGG CGC A**C**C CTA GAG GT**G** TTC AAG GAA CTC

                                  Thr (T)   Val (V)

Figure 23. Nucleotide and amino acid substitution of HCMV strain of 2F. A) gB1. B) UT1. The solid letters referred to nucleotide substitution. The number, which labeled on nucleotide sequences, showed amino acid position effecting amino acid substitution.

A) 6H gB1 sequences

                                        463
M60927 AAT AGA ACC AAA AGA AGT ACA AAT GGC AAC AAT GCA ACT CAT TTA
                                        Asn (N)
6HC13   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA
6HC16   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA
6HC21   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA
6HC25   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA
6HC12   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA
                                        Asp (D)

        502
M60927 CAA ATC GCA GAA GCC TGG TGT GTG GAT CAA CGG CGC TCC CTA GAG
            Ile (I)   Ser (S)
6HC13   CAA ATC GCA GAA GCC TGG TGT GTG GAT CAA CGG CGC **AG**C CTA GAG
6HC16   CAA ATC GCA GAA GCC TGG TGT GTG GAT CAA CGG CGC **AG**C CTA GAG
6HC21   CAA A**C**C GCA GAA GCC TGG TGT GTG GAT CAA CGG CGC **AG**C CTA GAG
            Thr (T)
6HC25   CAA ATC GCA GAA GCC TGG TGT GTG GAT CAA CGG CGC **AG**C CTA GAG
6HC12   CAA ATC GCA GAA GCC TGG TGT GTG GAT CAA CGG CGC **AG**C CTA GAG
                                                            Ser (S)

        518   523
M60927 GTC TTC AGG GAA CTC AGC AAG ATC
            Arg (R)   Ile (I)
6HC13   GTC TTC A**A**G GAA CTC AGC AAG ATC
6HC16   GTC TTC A**A**G GAA CTC AGC AAG ATC
6HC21   GTC TTC A**A**G GAA CTC AGC AAG ATC
6HC25   GTC TTC A**A**G GAA CTC AGC AAG ATC
6HC12   GTC TTC A**A**G GAA CTC AGC AAG A**C**C
            Lys (K)   Thr (T)

B) 6H gB3 sequences

                                497   518
M60933 TAT GAT ACG TTG CGC AGC TAC ATC AAT CGG GCG TTG GCG CAG ATC   AAG
                                    Arg (R)   Lys (K)
6HC4   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG GCG CAG ATC   A**G**G
6HC7   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG GCG CAG ATC   A**G**G
6HC14   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG GCG CAG ATC   A**G**G
6HC22   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG GCG CAG ATC   A**G**G
                                    Gln (Q)   Arg (R)

Figure 24. Nucleotide and amino acid substitution of HCMV strain of 6H. A) gB1. B) gB3.
The solid letters referred to nucleotide substitution. The number, which labeled on nucleotide
sequences, showed amino acid position effecting amino acid substitution.

A)   1I gB1 sequences

                               463

M60927 AAT AGA ACC AAA AGA AGT ACA AAT GGC AAC AAT GCA ACT CAT TTA

                                Asn (N)

1IC1   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA

1IC3   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA

1IC4   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA

1IC10   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA

1IC13   AAT AGA ACC AAA AGA AGT ACA **G**AT GGC AAC AAT GCA ACT CAT TTA

                                Asp (D)

     513   518

M60927 TCC CTA GAG GTC TTC GTC TTC AGG GAA CTC AGC AAG ATC

       Ser (S)   Arg (R)

1IC1   A**GC** CTA GAG GTC TTC GTC TTC A**A**G GAA CTC AGC AAG ATC

1IC3   A**GC** CTA GAG GTC TTC GTC TTC A**A**G GAA CTC AGC AAG ATC

1IC4   A**GC** CTA GAG GTC TTC GTC TTC A**A**G GAA CTC AGC AAG ATC

1IC10   A**GC** CTA GAG GTC TTC GTC TTC A**A**G GAA CTC AGC AAG ATC

1IC13   A**GC** CTA GAG GTC TTC GTC TTC A**A**G GAA CTC AGC AAG ATC

       Ser (S)   Lys (K)

B)   1I gB3 sequences

                449   458

M60933 GCC AAT AGC TCC GGT GTG AAC TCC ACG CGT AGA ACC AAG AGAAGT

              Ser (S)   Thr (T)

1IC12   GCC AAT AGC TCC GGT GTG AAC TCC ACG CGT AGA ACC AAG AGAAGT

1IC11   GCC AAT AGC TCC GGT GTG AAC TCC ACG CGT AGA ACC AAG AGAAGT

1IC2   GCC AAT AGC **C**CC GGT GTG AAC TCC ACG CGT AGA **T**CC AAG AGAAGT

              Pro (P)   Ser (S)

                                  497   518

M60933 TAT GAT ACG TTG CGC AGC TAC ATC AAT CGG GCG TTG   AAG

                                  Arg (R)   Lys (K)

1IC12   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG   A**GG**

1IC11   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG   A**GG**

1IC2   TAT GAT ACG TTG CGC AGC TAC ATC AAT C**A**G GCG TTG   A**GG**

                                  Gln (Q)   Arg (R)

Figure 25.   Nucleotide and amino acid substitution of HCMV strain of 1I. A) gB1. B) gB3. The solid letters referred to nucleotide substitution. The number, which labeled on nucleotide sequences, showed amino acid position effecting amino acid substitution.

5B-2 gB2 sequences

                                451

AD169   GCC AAT CGA TCC AGT CTG AAT ATC ACT CAT CGG ACC AGA AGA AGT
                                Leu (L)

5B-2C2 GCC AAT CGA TCC AGT **G**TG AAT ATC ACT CAT CGG ACC AGA AGA AGT
                                Val (V)

5B-2C6 GCC AAT CGA TCC AGT CTG AAT ATC ACT CAT CGG ACC AGA AGA AGT

5B-2C3 GCC AAT CGA TCC AGT CTG AAT ATC ACT CAT CGG ACC AGA AGA AGT

5B-2C1 GCC AAT CGA TCC AGT CTG AAT ATC ACT CAT CGG ACC AGA AGA AGT

5B-2C5 GCC AAT CGA TCC AGT CTG AAT ATC ACT CAT CGG ACC AGA AGA AGT

                                     486   489   491

AD169   CAC AAT CTG GTC TAC GCC CAG CTG CAG TTC ACC TAT GAC ACG TTG
                                 Phe (F)   Asp (D)   Leu (L)

5B-2C2 CAC AAT CTG GTC TAC GCC CAG CTG CAG TTC ACC TAT GAC ACG TTG

5B-2C6 CAC AAT CTG GTC TAC GCC CAG CTG CAG **C**TC ACC TAT GAC ACG TTG
                                Leu (L)

5B-2C3 CAC AAT CTG GTC TAC GCC CAG CTG CAG TTC ACC TAT **T**AC ACG TTG
                                     Tyr (Y)

5B-2C1 CAC AAT CTG GTC TAC GCC CAG CTG CAG TTC ACC TAT GAC ACG T **C**G

5B-2C5 CAC AAT CTG GTC TAC GCC CAG CTG CAG TTC ACC TAT GAC ACG T **C**G
                                           Ser (S)

Figure 26. Nucleotide and amino acid substitution of HCMV strain of 5B-2. The solid letters referred to nucleotide substitution. The number, which labeled on nucleotide sequences, showed amino acid position effecting amino acid substitution.

Codon:	449	450	451	453	455	456	457	458	459	462	463	464	465	466	467	468	469	470	471	472	473	474	477	479	480	486	489	491	492	493	497	500	502	513	518	522	523
gB1																																					
M60927	S	S	L	L	H	N	R	T	K	T	N	G	N	N	A	T	H	L	S	N	M	E	H	L	V	F	D	L	R	G	R	A	I	S	R	K	I
P1 (1)	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P2 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	K	-	-
P3 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	K	M	-
P4 (1)	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	T
P5 (1)	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	K	-	-
P6 (9)	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-
P7 (1)	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M
gB2																																					
AD169	S	S	L	I	H	X	R	T	R	T	S	D	N	N	T	T	H	L	S	S	M	E	H	L	V	F	D	L	R	G	R	A	I	T	K	K	I
P1 (3)	-	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P2 (1)	-	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-
P3 (1)	-	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-
P4 (1)	-	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-
P5 (1)	-	-	V	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-
gB3																																					
M60933	S	G	V	S	R	X	R	T	R	T	X	G	N	T	T	T	L	S	L	E	S	E	R	V	L	F	D	L	R	S	R	A	I	T	K	K	I
P1 (1)	-	-	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P2 (1)	-	-	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	Q	-	-	-	R	-	-
P3 (1)	-	-	-	-	S	X	-	-	-	-	X	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	R	-	-
P4 (1)	P	-	-	-	-	X	-	S	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	R	-	-
P5 (9)	-	-	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
P6 (5)	-	-	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
P7 (1)	-	S	-	A	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	R	-	-
P8 (2)	-	-	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	R	-	-
P9 (2)	-	-	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
UT1																																					
M60927	S	S	L	L	H	N	R	T	K	T	N	G	N	N	A	T	H	L	S	N	M	E	H	L	V	F	D	L	R	G	R	A	I	S	R	K	I
P1 (3)	-	-	-	-	-	S	-	-	-	A	D	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-
P2 (1)	-	-	-	-	-	S	-	I	-	A	D	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-
P3 (1)	-	-	-	-	-	S	-	-	-	A	D	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	T	K	-	-
P4 (1)	Q	G	V	H	R	S	X	-	-	K	Q	-	-	K	-	-	Q	-	P	E	R	-	R	V	L	-	-	-	-	S	-	-	-	T	K	-	-
UT2																																					
M60927	S	S	L	L	H	N	R	T	K	T	N	G	N	N	A	T	H	L	S	N	M	E	H	L	V	F	D	L	R	G	R	A	I	S	R	K	I
P1 (3)	-	-	-	-	-	S	-	A	-	A	D	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P2 (1)	-	-	-	-	-	S	-	A	-	A	D	-	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 27. Variable amino acid of HCMV gB segment between codons 446 and 523. -, no change from their reference strain; X, deletion at this location. Abbreviation of amino acids was shown in Appendix I.

PART II   Determination of HCMV UL97 mutation

1. Sensitivity of HCMV *UL97* PCR

The serial 10-fold concentration of HCMV AD169 DNA was used to define the sensitivity of the HCMV UL97 gene amplification method. The sensitivity of the PCR assay was found to be at least 10 pg of HCMV DNA (Figure 28).

2. Detection of HCMV *UL97* in clinical specimens

A total of 7 clinical specimens from 3 patients were included in this study. However, only 3 clinical specimens from 3 patients, which had HCMV load grater than 100,000 copies/ml, were *UL97* PCR positive (Table 19). One of them (4H), defined as mixed gB genotypes, were cloned before HCMV *UL97* sequencing. After cloning, 4 clones of 4H clinical sample were *UL97* PCR positive.
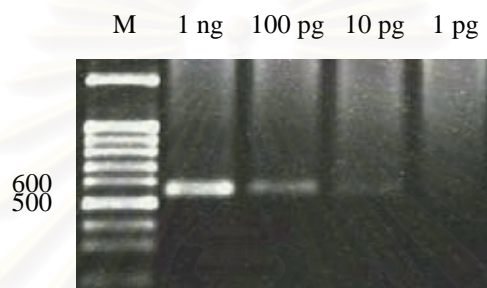
Figure 28. The sensitivity of the HCMV *UL97* PCR assay. Ten-fold concentration of 1 pg to 1 ng of the HCMV UL97 gene were amplified by PCR. Lane M, showed the DNA marker (bp).

Table 19. Characteristic of patients who received GCV drug over 3 months.

No.	Disease	Code	Date	HCMV load (copies/ml)	gB genotype	*UL97* PCR assay
27	KT	5F	15-7-02	115,000	3	+
		9I	12-12-02	12,100	2+3	-
29	KT	8A	18-6-03	4,330	2	-
		4A	30-9-03	3,600	2	-
		9F	23-2-04	>100,000	2	+
39	HIV	6B-2	24-4-03	2,410	2	-
		4H	9-7-03	>100,000	1+2+untype	+ *

KT, kidney transplant

* Clinical sample was cloned before *UL97* sequencing.

3. HCMV *UL97* sequencing

Partial UL97 sequences coding codon 436 to 610 were determined in AD169 strain (as positive control), 4 independent successive clones of 4H, and 2 clinical specimens (5F and 9F) of unique gB genotype.

The alignment of HCMV AD169 sequence from GenBank (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=28373214&itemID=90&view= gbwithparts) revealed 100% nucleotide identity and 100% peptide sequence homology to our control AD169 strain. The percentage of homology in nucleotide and peptide sequence of clinical strains comparing to AD169 strain was shown in Table 20. Although, 9F clinical sequence showed 99.21% nucleotide homology, there was 100% peptide sequence identity with strain AD169. On the other hand, 5F clinical sequence showed 97.23% nucleotide homology and 96.57% peptide sequence homology. Among 4 independent clones of 4H, 2 clones had same nucleotide sequence, demonstrated 99.21% nucleotide homology and 100% peptide sequence identity. The remaining of 4H cloning sequences (4HC5 and 4HC10) showed 98.81 % and 98.61 % nucleotide homology, respectively. Peptide sequence similarity of 4HC5 was 98.86% and 4HC10 was 99.43%.

Among the total of 6 sequences (5F, 9F, 4HC4, 4HC5, 4HC8 and 4HC10) from 3 clinical specimens (5F, 9F and 4H), 10 of 175 codons in UL97 showed sequence variation effecting peptide encoding (Figure 29). A Leu-to-Phe (or CTC to TTC) mutation at codon 446, Cys-to-Trp (or TGC to TGG) mutation at codon 453, a His-to-Pro (or CAC to CCA) mutation at codon 454, an Ala-to-Tyr (or GCC to TAT) mutation at codon 588, an Ala-to-Glu (or GCG to GAG) mutation at codon 590, a Thr-to-Lys (or ACG to AAG) mutation at codon 601, and an Asp-to-Glu (or GAC to GAG) mutation at 605 were found in 5F clinical sequence. From 2 UL97 cloning independent sequences of 4H clinical specimen, one clone (4HC5) had mutation at codon 496 (Val-to-Glu or GTG to GAG), and codon 512 (Ser-to-Pro or TCG to CCG). Another (4HC10) had a Thr-to-Ala (or ACG to GCG) mutation at codon 502. However, all of UL97 mutations found in this study were not related to UL97 position conferring GCV resistant as previously reports.

Table 20. Nucleotide and amino acid sequence homology of UL97 coding sequence of clinical strains.

Code of Sequence	Sequence homology (%)*	
	Nucleotide	**Amino acid**
5F	97.23	96.57
9F	99.21	100
4HC4	99.21	100
4HC5	98.81	98.86
4HC8	99.21	100
4HC10	98.61	99.43

* Sequence homology was compared to UL97 of HCMV strain AD169

```
           446                            453  454
AD169  CTC AAT CAC CAG TGT CGT GTA TGC CAC TTT GAC ATT ACA CCC ATG
       Leu (L)                          Cys (C) His (H)
9F     CTC AAT CAC CAG TGT CGT GTA TGC CAC TTT GAC ATT ACA CCC ATG
4HC4   CTC AAT CAC CAG TGT CGT GTA TGC CAC TTT GAC ATT ACA CCC ATG
4HC8   CTC AAT CAC CAG TGT CGT GTA TGC CAC TTT GAC ATT ACA CCC ATG
4HC5   CTC AAT CAC CAG TGT CGT GTA TGC CAC TTT GAC ATT ACA CCC ATG
4HC10  CTC AAT CAC CAG TGT CGT GTA TGC CAC TTT GAC ATT ACA CCC ATG
5F     TTC AAT CAC CAG TGT CGT GTA TGG CCA TTT GAC ATT ACA CCC ATG
       Phe (F)                          Trp (W) Pro (P)
            496                      502
AD169  GTG GCC GTC TTT CAG GAG ACG GGT ACG GCG CGC CGC ATC CCC AAC
       Val (V)                     Thr (T)
9F     GTG GCC GTC TTT CAG GAG ACG GGC ACG GCG CGC CGC ATC CCC AAC
4HC4   GTG GCC GTC TTT CAG GAG ACG GGC ACG GCG CGC CGC ATT CCC AAC
4HC8   GTG GCC GTC TTT CAG GAG ACG GGC ACG GCG CGC CGC ATT CCC AAC
4HC5   GAG GCT GTC TTT CAG GAG ACG GGC ACG GCG CGC CGC ATC CCC AAC
       Glu (E)
4HC10  GTG GCC GTC TTT CAG GAG GCG GGC ACG GCG CGC CGC ATT CCC AAC
                              Ala (A)
5F     GTG GCC GTC TTT CAG GAG ACG GGC ACG GCG CGC CGC ATC CCC AAC


            512
AD169  TGC TCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCC
       Ser (S)
9F     TGC TCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCC
4HC4   TGC TCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCC
4HC8   TGC TCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCC
4HC5   TGC CCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCC
            Pro (P)
4HC10  TGC TCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCC
5F     TGC TCG CAC CGT CTG CGC GAA TGT TAC CAC CCT GCT TTC CGA CCT


             588     590
AD169  AAG CAC GCC GGC GCG GCC TGC CGC GCG TTG GAG AAC GGT AAG CTC
            Ala (A)   Ala (A)
9F     AAG CAC GCC GGC GCG GCC TGC CGC GCG TTG GAG AAC GGC AAG CTC
4HC4   AAG CAC GCC GGC GCG GCC TGC CGC GCG TTG GAG AAC GGC AAG CTC
4HC8   AAG CAC GCC GGC GCG GCC TGC CGC GCG TTG GAG AAC GGC AAG CTC
4HC5   AAG CAC GCC GGC GCG GCC TGC CGC GCG TTG GAG AAC GGC AAG CTC
4HC10  AAG CAC GCC GGC GCG GCC TGC CGC GCG TTG GAG AAC GGC AAG CTC
5F     AAG CAC TAT GGC GAG GCC TGC CGC GCG TTG GAG AAC GGT AAG CTC
            Tyr (Y)   Glu (E)
           601         605
AD169  ACG CAC TGC TCC GAC GCC
       Thr (T)      Asp (D)
9F     ACG CAC TGC TCC GAC GCC
4HC4   ACG CAC TGC TCC GAC GCC
4HC8   ACG CAC TGC TCC GAC GCC
4HC5   ACG CAC TGC TCC GAC GCC
4HC10  ACG CAC TGC TCC GAC GCC
5F     AAG CAC TGT TCC GAG GCC
       Lys (K)      Glu (E)
```

Figure 29.   Nucleotide and amino acid substitution of HCMV UL97. The solid letters referred to nucleotide substitution. The number that labeled on nucleotide sequences showed amino acid position effecting from nucleotide mutation.

CHAPTER VI

DISCUSSION

HCMV infection is generally asymptomatic in the immunocompetent individual although the virus persists in the host for life. However, severe infection with different clinical manifestations is common in immunocompromised patients with AIDS or chronic diseases and transplant recipients. Previously, several reports have suggested an association of different gB genotypes with pathology (4, 27, 30, 31, 37, 38) since gB is considered to be a multifunctional envelope component responsible for virion entry, cell to cell spread, syncytium formation and is the major target for neutralizing antibodies (23-26). HCMV gB genotyping is based on the variation of proteolytic cleavage site between amino acid 460 and 461. Therefore, at least 4 gB genotypes were determined by using PCR and RFLP.

In this study, HCMV gB genotyping was done in 161 clinical specimens obtained from King Chulalongkorn Memorial Hospital during the year of 2000 to 2004. They were all positive for HCMV-DNA determined by in-house or Amplicor PCR assay. Only 70.19 % (113/161) were successfully typed (Table 7). Among those 113 samples, mixed gB genotype (34.51 %) was the most prevalent found, following by gB1 (32.74 %), gB3 (15.04 %) and gB2 (10.62 %). None of gB4 was determined and 7.08 % were untyped (Table 8). Mixed gB2+gB3 and MUT genotype (30.77 %) are the most common (Table 9). In agreement with previous reports (28, 32, 36, 40, 173, 174, 176-182), HCMV infection with genotype gB1, gB2 and gB3 was relatively common and that infection with gB4 was uncommon. Although some studies found a greater predominance of other gB types (gB2+gB3) (35, 36, 173), differences in genotype frequencies may, in part, be due to variation in study population as well as in the geographical distribution of HCMV genotype (Table 4.) Interestingly, 2 untype HCMV gB genotype (UT1 and UT2) identified in this present study, one (UT2) had similar restriction pattern as previously report (27), whereas another UT1 has never been published anywhere else.

Since HCMV associated with various types of diseases, the clinical specimens may be obtained from different site of the body. Our results confirmed these previous reports of possible detection of HCMV-DNA in various types of specimens, e.g. blood, urine, CSF, WBC, tissue, and BAL, etc. The prevalence of gB genotypes differed if samples were obtained from different sites as well as the presence of more than single gB genotype in sample of the same patient (Table 10). This observation was similar to other reports (27, 28, 33, 35, 39). Mixed gB genotypes was found predominantly in plasma (36.78 %) and WBC (29 %) (Table 8). Coaquette, A. et al hypothesized that leukocytes might allow a switch from one genotype to another during the progression of the disease and in agreement with this hypothesis, leukocytes are one of the major HCMV reservoirs (36).

A high proportion of patients (more than 90 %) was reported to be infected one gB genotype (4, 27, 28, 31-42, 174-178, 181, 182), corresponding to our result, although it was slightly lower (65.5 %, Table 11). Mixed infection was also demonstrated in 36 (37.50 %) of patients (Table 11). The different patterns of gB genotype were observed among samples collecting at different time of 5 mixed HCMV infected patients but there was common gB genotype found among those samples (Table 10). This phenomenon may be involved reinfection of new strain or viral immune evasion from NAb or recombination of existing strain with the new viral strain.

In this present study, we compare the frequency distribution of gB genotype between male and female and no difference was found (Figure 15, Table 13). Moreover, 2 groups of immunocompromised (HIV infected patients and transplant patients) were also compared the prevalence of gB genotype. The result showed that gB1 (61.54 %) was the most common type among transplant patients, while mixed gB genotypes infection was dominantly in HIV infected patients (Figure 16, Table 14). Many observations reported the high prevalence of gB2 in HIV infected patients (27, 29-31, 41, 42, 174, 175), which opposed to our data: none of gB2 was determined (Table 14). While, our result of high gB1 in transplant patients was the same as other studies (4, 27, 33, 34, 38, 179). The high mixed HCMV infection within HIV infected patients was not unexpected. Since the immune response in these groups of patients was very poor, the reactivation or reinfection of HCMV could often repeatedly occur. Thus, the mixed gB genotype should be the result of such phenomenon. If the gB genotypes play role in severity of the HCMV disease, it should effect the HCMV viral

load. However, no association of these 2 factors (gB genotypes and HCMV viral load) was demonstrated (Figure 17).

In attempt to search for gB4, mixed gB3+gB4 specimen was amplified and cloned. Interestingly, untyped gB genotype was cloned together with gB3 and combination of these 2 genotypes showed restriction pattern similar to gB3+gB4 (Table 15, Figure 18). Two samples of MUT were also done (Table 15). One sample (6H) contained 2 genotypes (gB1 and gB3) while another sample (1I) consisted of 3 genotypes (gB1, gB2 and gB3) (Figure 19, 20). Therefore, MUT samples may be the combination of 2 or more gB genotypes. From these information, the gB genotype determined by PCR-RFLP, was quite limited and possibly misinterpreted especially in sample with mixed HCMV infection. Then, DNA sequencing method was performed using different unique gB genotypes as well as cloned gB genotypes. Surprisingly, one samples (5D) had only 77.12 % nucleotide homology to gB1 while percentage of nucleotide homology was 96.96 % closed to gB3 (Table 16). Similar observation was found in other 5 samples of gB2 (Table 16). Phylogenetic tree analysis has also been performed (Figure 22). The explanation of this finding is usually possible, because RFLP based method depends on the only 9 nucleotide sequences (*Hin*f I; G'AnTC, *Rsa* I; GT'AC), whereas sequencing based method depends on all of nucleotide sequences that were analyzed. One out of 5 controversial sequences, 5D sequence had mutation at *Hin*f I and *Rsa* I restriction site resulting in restriction fragment length, were similar to gB3 pattern (Figure 21). That probably involves DNA population conducting to PCR reaction, if 5D sample really has mixed gB genotypes. Even so, that assumption could not be proved because 5D DNA was not enough to do the cloning.

In addition, most of both UT1 and UT2 sequences showed to be clustered in HCMV gB1 (Figure 22, Table 17). While, 1 out of 6 UT1 (9D) was a new sequence variant that less closely matched with the known of 4 gB genotypes (80.9 % - 87.8 %), as shown in Table 17. Sequence homology among HCMV strains within each group of the present study was more than 97 % and gB sequence similarity between strains of differing group was about 71 % - 75 %, as shown in Table 18. According to previous reports, nucleotide homology between clinical strains within each genotype was 98.6 % - 99.9 %, while homology between strains of differing genotypes was 88 % - 95 % (18, 28). Moreover, gB3 was the most divergent (76.9 % - 79.6 %) as shown in Table 18, while gB1 had about 92 % homology with gB2 and gB4.

Our data clearly demonstrated the presence of intragenotypic variation between HCMV strains from different patients (Table 16) as well as within the same patient (Table 16, Figure 23-26). The difference in percentage of nucleotide homology was less than 1 % suggesting that mutation rather than reinfection was the common mechanism in inducing the intragenotypic variation. Some of nucleotide variations will result in either changing or unchanging amino acid type, as summarized in Figure 27. Changing in amino acid residue will further effect the conformation of polypeptide. Since gB plays important role in induction of NAb, conformational change of the gB molecule may be one mechanism of escape immune response (NAb). Location of the neutralizing epitope of gB has previously been reported (63, 118). Amino acid residues between 476 and 645 was suggested to be the major immunodominant neutralizing domain. Our results indicated the variably of amino acid in such region. From all data presented here convincing us that intragenotypic variation in a patient was caused by positive selection of host immunity. The same observation has been reported in many viruses by other studies (183-186).

Since nowadays, GCV is the most widely used for treatment of HCMV associated diseases and preemptive treatment of infection. Several reports revealed that HCMV UL97 open reading frame codes for a proteinkinase homologue capable of phosphorylating GCV to be active form (44, 45). Acquisition of mutations in UL97 phosphotransferase appears to be a crucial step in the selection of GCV resistant. Although, the mutations of UL97 could be detected by restriction enzyme polymorphisms (47, 52, 53, 149-153), sequencing is now the method of choice because not all mutations affect restriction enzyme recognition sequences (46, 47, 49, 52-54, 140, 145-147, 150, 153, 154).

In this study, UL97 mutations were determined by sequencing based method. The requirement for high quantity of HCMV DNA (up to 10 pg, Figure 28) was the limitation of UL97 amplification for sequencing. Usually examination of UL97 mutation was done after passaging HCMV isolates into tissue culture (44, 47, 49, 50, 53). Requiring of fresh specimen (WBC) was the necessity for HCMV propagation. All specimens obtained in this study were frozen plasma, which were not suitable for performing isolation. Therefore, only 3 out of 7 clinical samples from 3 patients were UL97 PCR positive. According to UL97 sequencing, all of 6 UL97 sequences composing of 5F, 9F and 4H independent cloning sequences (4HC4, 4HC5, 4HC8

and 4HC10) had nucleotide and peptide sequence homology, when they were compared with HCMV AD169, approximately 97.2 % - 99.2 % and 96.5 % - 100.0 %, respectively (Table 20). Among 8 amino acid substitutions, 8 out of 10 codons (codon 446, 453, 454, 496, 502, 512, 588 and 605) were novel positions that have never been reported (Figure 29, Table 2). Although, the remaining 2 codons (codons 590 and 601), associated with HCMV GCV resistance for Thr substitution at codon 590 (Ala 590 Thr) and Met substitution at codon 601 (Thr 601 Met) (141), here they were substituted with Glu (Ala 590 Glu) and Lys (Thr 601 Lys) instead. From our results, we can not conclude whether these viruses were GCV resistance based on these 10 UL97 mutations. The phenotypic antiviral drug was the method of choice to confer the resistant properties. Unfortunately, this technique requires infectious virus and we are unable to propagate from these specimens.

In summary, this study demonstrated the prevalence of HCMV gB genotype in Thai patients and gB1 was the most common genotype. Mixed gB genotypes was found often in HIV infected patients. There was no sexual preponderance in distributing HCMV gB genotype and no association between gB genotype and HCMV viral load was indicated. We also confirmed that DNA sequencing assay was better than PCR-RFLP assay. Moreover, inter and intragenotypic variations among HCMV gB genotype were illustrated. It was clearly showed gB3 genotype was divergent distinctively from other types. The intragenotypic variation within a patient suggesting, positive selection of host immunity induced by mutation. However, reinfection of new types may not be excluded. An attempt to search for GCV resistant strain was failed due to limitation of samples, even though 8 novel positions of mutation were found.

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APPENDICES

APPENDIX I

PATIENTS DATA

No.	Code	Sex	Type of patient	Sample	HCMV load	gB* genotype	No.	Code	Sex	Type of patient	Sample	HCMV load	gB* genotype
1	1A	M		plasma		1 [a]	45	9H	M		plasma	26,800	1
2	2A	M		tissue		1	46	1I	F		plasma	>100,000	MUT
3	3A	M	HIV	plasma		1	47	4I	F		plasma		1+2
4	1B	M	Tx	CSF		1	48	5I	F	KT	plasma	2,290	1
	2B			sigmoid		1 [a]	49	6I	F		plasma	48,600	2+3+UT
	3B			duodenum		1	50	7I	M		plasma		2 [a]
5	4B	F		CSF		2+3	51	8I	M		plasma	5,270	3 [a]
6	5B	M		BAL		MUT	52	5A	F		plasma	10,700	MUT
7	6B	F	HIV	plasma		3	53	6A	F		plasma	4,960	1
8	7B	F	HIV	CSF		1	54	7A	M		plasma	8,540	2+3
9	9B	M	KT	plasma		1	55	9A	M		plasma	3,250	3+4
10	1C	M		WBC		1	56	1A-2	F	HIV	plasma		MUT
11	2C	F		plasma		3 [a]	57	3A-2	F	HIV	plasma		MUT
	3C			WBC		3	58	4A-2	M	HIV	plasma		2+3
12	4C	M		plasma		1	59	5A-2	M	HIV	plasma		1
	5C			WBC		1	60	2B-2	M		plasma	1,610	MUT
13	6C	M	Tx	plasma		1	61	3B-2	M		plasma	561	2+3
	7C			WBC		1 [a]	62	4B-2	F	BMT	plasma	791	1 [a]
14	8C	M		WBC		1	63	5B-2	M		plasma	2,220	2
15	9C	F		plasma		2 [a]	64	7B-2	M	HIV	plasma	1,210	3 [a]
16	1D	F		plasma		1	65	10B-2	M	HIV	liver biopsy		1
17	3D	M	HIV	plasma		3 [a]	66	3C-2	F	HIV+TB	plasma	7,350	UT2
18	5D	F	Tx	plasma		1 [b]	67	7C-2	F	HIV	plasma		1
19	6D	M		plasma		MUT	68	2D-2	F	HIV	plasma		3+4+UT
20	9D	F	HIV	plasma		UT1	69	5D-2	M	HIV	plasma		UT2
21	2E	F	BMT	throat washing		2 [b]	70	6D-2	M	HIV	plasma		3
	3E			BAL		2	71	7D-2	M	HIV	plasma		UT2
	6F			plasma	51,800	1+2	72	1E-2	F		plasma	24,500	UT2
22	5E	M	KT	plasma		3 [a]		5E-2			plasma	7,760	UT2
23	9E	M	KT	plasma	>100,000	1 [a]	73	2E-2	F	HIV	plasma	>100,000	1
24	1F	F	KT	plasma	>100,000	1	74	3E-2	F		plasma	>100,000	3
25	2F	F	HIV	plasma	>100,000	3+4	75	4E-2	F	HIV	plasma	>100,000	3+4
26	3F	M	HIV	plasma	62,700	1 [a]		8E-2			plasma	6,130	3+4
27	4F	F		plasma	36,500	1+2	76	6E-2	F		plasma	80,600	UT1
28	5F	M	Renal Tx	plasma	>100,000	3	77	7E-2	M		plasma	>100,000	2+3
				plasma	12,100	2+3	78	9E-2	M	HIV	plasma	2,682	3 [a]
29	2I	M	HIV	plasma	15,500	1+4	79	10E-2	F		plasma	15,200	1
	8F			plasma	3,840	1+2	80	2F-2	M	HIV	plasma	11,300	1
	3I			plasma	18,200	MUT	81	7H-2	F		CSF		1
30	8A	F	KT	plasma	4,830	2 [b]	82	10H-2	M	HIV	plasma	5,740	3
	4A			plasma	3,600	2		2I-2			tissue		2+3
	9F			plasma	>100,000	2	83	1I-2	M		plasma	>100,000	1
31	2G	F	HIV	plasma	88,700	UT2	84	3I-2	M		plasma	16,700	1+2
32	5G	F	HIV	plasma	18,500	3	85	4I-2	F		plasma	>100,000	2+3
33	6G	M		plasma	>100,000	MUT	86	5I-2	F	BMT	plasma	635	2
34	7G	M		plasma	14,900	2+3	87	6I-2	M		plasma	9,070	1
35	8G	F		plasma	16,200	1+3	88	3J-2	F		plasma	22,200	3
36	9G	M		plasma	68,700	1		8J-2			plasma	8,450	3

APPENDIX I (CONTINUED)

PATIENTS DATA

No.	Code	Sex	Type of patient	Sample	HCMV load	gB* genotype	No.	Code	Sex	Type of patient	Sample	HCMV load	gB* genotype
37	1H	F		plasma	45,000	1	89	4J-2	M	HIV	plasma	>100,000	1 [a]
38	2H	F		plasma	55,300	1	90	2K-2	F		plasma	12,900	1+4
39	3H	M		plasma	16,500	MUT	91	5K-2	M		plasma	50,900	2 [b]
40	4H	M	HIV	plasma	>100,000	1+2+UT	92	6K-2	F		plasma		2+3
	6B-2			plasma	2,410	2 [b]	93	7K-2	F		plasma	39,900	3 [a]
41	5H	F		plasma	14,900	2+3	94	8K-2	F		plasma	10,600	2
42	6H	F	HIV	plasma	42,100	MUT	95	10K-2	F		plasma	10,700	3
43	7H	F	HIV	plasma	22,400	2+3	96	3L-2	M		plasma	>100,000	1
44	8H	M	BMT	plasma	3,400	1+2							

*   gB genotype by PCR-RFLP

[a]   gB genotype by DNA sequencing were in agreement with PCR-RFLP

[b]   gB genotype by DNA sequencing definded to be gB3

APPENDIX II

ONE AND THREE LETTERS SYMBOLS FOR THE AMINO ACIDS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leusine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

APPENDIX III

REAGENTS, MATHERIALS AND INSTRUMENTS

A. Media and Reagents

Absolute ethanol	(Merck, Germany)
AccuGel™ 19:1	(National Diagnostics, U.S.A.)
Acetone	(Merck, Germany)
Agarose	(Bio Basic, U.S.A.)
Ammonium persulphate	(Pharmacia Biotech, Sweden)
Ampicilin	(Sigma, U.S.A.)
Boric acid	(Sigma, U.S.A.)
100 bp DNA Ladder	(New England Bio Labs, U.S.A.)
DMSO	(Sigma, U.S.A.)
5-bromo-4-chlor-3-indolyphosphate/ nitroblue tetrazolium	(Bio-Rad, U.S.A.)
Bromphenol blue	(USB, U.S.A.)
dNTPs	(Promega, U.S.A.)
Ethylenediamine tetraacetic (EDTA)	(Amreso, U.S.A.)
Ethydium bromide	(Bio-Rad, U.S.A.)
Fetal bovine serum	(GIBCO BRL, U.S.A.)
IsoPrep	(Robbins Scientific, Norway)
Glycerol	(USB, U.S.A.)
Hinf I	(New England Bio Labs, U.S.A.)
NaCl	(Merck, Germany)
Rsa I	(New England Bio Labs, U.S.A.)
Taq DNA polymerase (with MgCl2 and PCR buffer)	(Promega, U.S.A.)
TEMED	(USB, U.S.A.)
Tris-base	(Sigma, U.S.A.)
Tryptone	(Difco, U.S.A.)
Xylence cyanol	(Sigma, U.S.A.)
Yeast extract	(Difco, U.S.A.)

B. Materials

Filter Tip	(Sorenson, U.S.A.)
Microcentrifuge tube	(Sorenson, U.S.A.)

C. Instruments

Autoclave (model-SS-325)	(Tomy, Japan)
Chemi doc	(Bio-Rad, U.S.A.)
DNA thermocycle system	(Hybaid, U.S.A.)
Electrophoresis chamber	(CBS, U.S.A.)
Microcentrifuge	(Fotodyne, U.S.A.)
Mixer-Vertex-Genic	(Scientific industries, U.S.A.)
Power supply (Model 1000/500)	(Bio-Rad, U.S.A.)
Refrigerator	(Toshiba, Japan)
Spectrophotometer (SmartSpect™ 3000)	(Bio-Rad, U.S.A.)
Vertical electrophoresis chamber	(CBS, U.S.A.)
Water bath	(Julabo, Germany)

APPENDIX IV

REAGENTS PREPARATION

Reagent for Cloning of HCMV DNA

1. Luria-Bertani broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
1 M NaOH	1 ml
Distilled water to	1 L

Sterilized by autoclaving 121 ℃ 15 minutes

2. Luria-Bertani agar plate

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
1M NaOH	1 ml
Distilled water to	1 L

Sterilized by autoclaving 121 ℃ 15 minutes

To pour plates, agar was allowed to cool about 50°C and then ampicillin 100 mg/ml was added. After drying, plates were stored at 4°C until used.

3. 1 M CaCl$_2$

CaCl$_2$	11.1 g
Distilled water	100 ml

Sterilize by autoclaving 121 ℃ 15 minutes

4. **Ca/Glyceral (15 % glyceral)**

1 M CaCl$_2$	5	ml
Glyceral	15	ml
Distilled water	80	ml

Sterilize by sterile filtration

5. **0.5 M EDTA**

Na$_2$EDTA·2H$_2$O	18.61	g
Distrilled water to	100	ml

Adjust the pH to 8.0 with 10 M NaOH

Sterilize by autoclaving 121 ℃ 15 minutes and store at room temperature

6. **2 M Tris-Cl, pH 7.4**

Tris base	24.22	g
Concentrated HCl	14	ml
Distrilled water to	100	ml

Sterilize by autoclaving 121 ℃ 15 minutes

5. **TE buffer (Tris/EDTA)**

2 M Tris-Cl, pH 7.4	0.5	ml
0.5 M EDTA	20	μl
Distrilled water	99.48	ml

Sterilize by autoclaving 121 ℃ 15 minutes and store at room temperature

Reagent for Sequence

1. **70 % Ethanol**

Absolute Ethanol	70	ml
Sterile distilled water	30	ml

Store at -20 ℃

2. **125 mM EDTA**

Na$_2$ EDTA.2H$_2$O	2.326	g

Distilled water to	50	ml

Adjust the pH to 8.0 with 10 M NaOH

Sterilize by autoclaving 121 ℃ 15 minutes and store at room temperature

Reagent for Electrophoresis

1. 10 X Tris-borate buffer (TBE)

Tris-base	60.50	g
Boric acid	30.85	g
$Na_2EDTA \cdot 2H_2O$	3.72	g
Distilled water	1	L

Sterilize by autoclaving 121 ℃ 15 minutes and store at room temperature

2. Ethidium bromide (10 mg/ml)

Ethidium bromide	1	g
Sterile distilled water	100	ml

3. Loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Glycerol	30	g
Distilled water	69.5	ml

4. 1.5 % Agarose gel

Agarose	0.325	g
0.5 X TBE buffer	35	ml

5. 7 % Acrylamide gel

40% Acrylamide (AccuGel™ 19:1)	1.75	ml
DDW	7.15	ml
10X TBE	1	ml
10% ammonium persulphate	100	μl
TEMED	16	μl

BIOGRAPHY

Miss Suwimon Chantaraarphonkun was born on June 20, 1981 in Bangkok, Thailand. She previously graduated with the Bachelor degree (2nd Class Honour) of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand in 2002.