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ANALYSIS OF HUMAN CYTOMEGALOVIRUS IN THE HEALTHY HUMAN CARRIER

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A thesis submitted in partial fulfillment of the requirements
of the Open University for the degree of Doctor of Philosophy

December 1992

ADDENBROOKE'S HOSPITAL, CAMBRIDGE, U.K.
IN COLLABORATION WITH
THE UNIVERSITY OF CAMBRIDGE, CAMBRIDGE, U.K.

Author's number : PI 23039

Date of submission : December 1992

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ABSTRACT

Much circumstantial evidence has pointed to peripheral blood leukocytes as one site of persistence of Human Cytomegalovirus (HCMV) in healthy carriers. However, the exact population of peripheral blood cells that carry HCMV and to what extent they express HCMV gene products is not known. I have examined the sites of HCMV persistence in the peripheral blood of healthy carriers. Analysis of pure cell populations by the use of the polymerase chain reaction (PCR), sensitive to between 1 and 10 copies of the HCMV genome, showed that the predominant site of persistence was the monocyte. In addition, analysis of healthy seronegative subjects revealed that a significant number (30%) also harbored HCMV. Finally, study of granulocytes demonstrated no evidence of persistent HCMV.

Expression of HCMV during persistence was also analyzed, by using reverse transcription PCR (RT-PCR) with a sensitivity of between 1 and 100 infected fibroblasts. RNA from monocytes showed no evidence of polyadenylated immediate early (IE) or late transcripts. In contrast, *in vitro* differentiated monocyte-derived macrophages (MDM) did show evidence of HCMV gene expression with the class of HCMV genes expressed dependent on the method of differentiation. MDM treated with hydrocortisone (HC) and phorbol 12-myristate 13-acetate, expressed only IE1, but not IE2, glycoprotein B (gB) or phosphoprotein 28 (pp28) transcripts. Whereas, MDM treated with granulocyte-macrophage colony stimulating factor and HC expressed IE1, IE2 and gB, but not pp28 transcripts. In both cases, cocultivation experiments did not show plaques. Therefore, in the healthy carrier, persistence of HCMV in monocytes is independent of HCMV lytic gene expression, but *in vitro* differentiation of monocytes to MDM induced endogenous HCMV transcription consistent with the known permissivity of *in vivo* differentiated macrophages to HCMV infection.

ACKNOWLEDGEMENTS

I would like to thank Dr. John Sinclair and Professor J. G. P. Sissons for providing direction and support during the course of my work. In addition, I would like to thank all the members of the laboratory for their help and support. Specifically, I'd like to acknowledge Sandra Webb for her instruction of multiple techniques, Roger Stark for his invaluable assistance with the FACS, Graham Hayhurst for his assistance with the granulocyte work, and Joan Ballie and Linda Bryant for their guidance. I am particularly indebted to Carolyn Tysoe for donating the primers and probe for the gB polymerase chain reaction.

I am grateful to those people who kindly donated reagents for use in this project. They are individually acknowledged within the text.

To Jim and Sean

PREFACE

This dissertation is the result of my own work and includes nothing which is the outcome of work done by others.

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ABBREVIATIONS

Additional abbreviations to those defined in the text

A	adenine
ATCC	American Type Culture Collection
C	cytosine
CPE	cytopathic effect
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dT	deoxynucleotide thymine
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ETOH	ethanol
FACS	fluorescence-activated cell sort
FCS	fetal calf serum
G	guanine
GM-CSF	granulocyte macrophage colony stimulating factor
HC	hydrocortisone
HCMV	human cytomegalovirus
HELF	human embryonic lung fibroblasts
IE	immediate early
kd	kilodalton
KAW	potassium, acetate, and water buffer
Mab	monoclonal antibody
MDM	monocyte-derived macrophages
MEM	minimum essential medium
MOPS	3-(N-morpholino)propane sulfonic acid

Abbreviations

PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocytes
RBC	red blood cells
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute tissue culture media 1640
SSC	standard saline citrate
T	thymine
T150	150 cm ² flask
T75	75 cm ² flask
T25	25 cm ² flask
<i>Taq</i>	<u>Thermus aquaticus</u> polymerase
WBC	white blood cell

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Some of the earliest reports, of what was later determined to be human cytomegalovirus disease, examined a fatal congenital disseminated form of unknown infection (Jesionek & Kiolemenoglou, 1904; Ribbert, 1904). In the histologic examination of tissues from these cases, cellular enlargement with intranuclear inclusions were noted and the disease was named cytomegalic inclusion disease (CID). Historically, these fatal infections were thought to be due to a syphilitic agent or a protozoan and, in accordance with the latter etiology, one group, in 1910, proposed the name *Entamoeba mortinatalium* for the causative agent. Eventually, however, Goodpasture and Talbot, (1921) postulated that the swollen cells were actually host cells that were injured by a virus. Two pieces of evidence, in particular, supported this. First, the observation that cellular inclusions in CID tissues were similar to those seen in varicella skin lesions (Tyzzer, 1906; Lipschutz, 1921) was noted. Second, guinea pig disease transmitted from filtered guinea pig salivary gland extracts was reported (Cole & Kuttner, 1926). Eventually, human cytomegalovirus (HCMV) was able to be replicated in vitro (Rowe *et al.*, 1956 Smith, 1956; Weller *et al.*, 1957), allowing the true nature of the unknown agent to become clear and permitting studies of HCMV clinical infections. The name cytomegalovirus (Weller *et al.*, 1957) originated from the effects produced in tissue culture and, thus, the virus gets its name from this cytopathic effect.

1.2 HERPESVIRUS

1.2.1 Classification, Viral Structure and Organization of the Genome

In general, Herpesviruses have a distinct virion architecture with a central core containing double-stranded DNA. This is surrounded by an icosahedral capsid, which is coated with an asymmetric, amorphous tegument and an envelope, containing viral glycoproteins. The family Herpesviridae is classified into the subfamilies Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae (Roizman *et al.*, 1981) on the basis of biologic properties. Cytomegaloviruses are grouped as betaherpesviruses due to their long replication cycle, their species specificity and their cytopathic effects. The subfamily contains the genera Cytomegalovirus (HCMV) and Muromegalovirus (murine cytomegalovirus). In addition, the newly discovered human herpes virus 6 (Salahuddin *et al.*, 1986) has been classified as a betaherpesvirus and shows a close phylogenetic relationship to the genome of HCMV (Lawrence *et al.*, 1990; Thomson *et al.*, 1991).

HCMV is the largest known human herpesvirus and contains a 50% more complex genome than the alphaherpesvirus, herpes simplex. This is thought to be due to various gene acquisitions and duplications as well as sequence divergence. The large double stranded DNA genome of HCMV is present in the form of a torus in the core of the mature virion. It is organized into long and short unique sequences flanked by terminal repeats that are present internally in an inverted orientation and juxtaposed (Figure 1.1). The small direct terminal repeats are thought to be involved in cleavage and packaging and appear to be conserved among herpesviruses (Mocarski & Roizman, 1982; LaFemina & Hayward, 1983; Spaete & Mocarski, 1985; Marks & Spector, 1988). The internal repeats divide the genome, such that the unique portions are known to invert relative to each other leading to isomerization. Equimolar portions of these

isomers are present in virions extracted from infected cells. HCMV is the only betaherpesvirus with an isomerizing genome.

1.3 GENE EXPRESSION AND REPLICATION

HCMV gene expression during productive infection is coordinately regulated and sequentially ordered (DeMarchi, 1981; Wathen & Stinski, 1982; McDonough & Spector, 1983). Viral gene expression can be divided into three different kinetic classes (Figure 1.2). The first proteins to be synthesized, α or immediate-early, regulate transcription of the second group of HCMV proteins, the β or early proteins (Chang *et al.*, 1989; Spector *et al.*, 1990) as well as transactivate cellular genes (Dudding *et al.*, 1989; Santomenna & Colberg-Poley, 1990; Hagemeyer *et al.*, 1992a) and heterologous viral genes (Davis *et al.*, 1987; Tevethia *et al.*, 1987; Walker *et al.*, 1992). During the early phase, a number of regulatory proteins and viral enzymes are synthesized. These early proteins allow virion DNA replication to proceed. The origin of DNA replication during lytic infection has recently been defined (Hamzeh *et al.*, 1990) and is located in the center of the U_L between 0.35 to 0.40 map units. After DNA replication, the late or γ phase of replication proceeds. The late proteins consist primarily of viral structural components. The expression of these genes leading to production of virions occurs over a period of 72 hours in fully permissive cells in vitro.

1.3.1 Immediate Early Genes

The immediate early (IE) period follows virus entry into the cell and is independent of de novo viral protein synthesis such that they are still expressed in the presence of cyclohexamide. The IE RNA of HCMV arises from at least 3 regions of the HCMV genome (DeMarchi, 1981; Wathen *et*

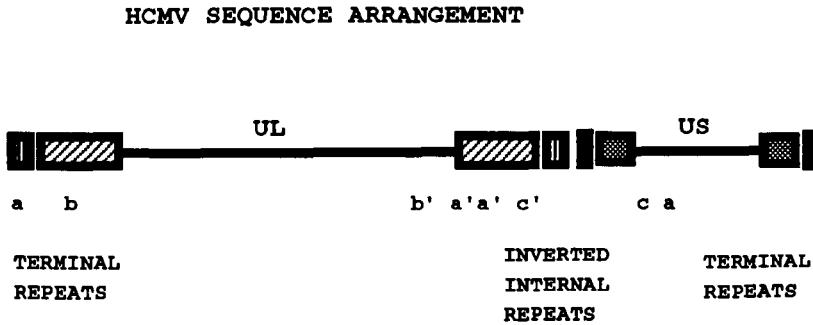


Figure 1.1. Genome Arrangement of Human Cytomegalovirus. The sequence arrangement shown here is that of the group E herpesviruses (Roizman, 1990) and is seen in both HCMV and herpes simplex viruses. One terminus has several copies of sequence **a** (designated by an open rectangle with vertical lines) next to a longer sequence **b** (shown by an open rectangle filled with diagonal lines) and the other terminus has a single copy of **a** (designated by the closed rectangle) and a more internal sequence **c** (shown by an open rectangle filled with cross-hatched lines). Sequences from both termini are repeated in an inverted orientation (inverted sequences shown as primes) and juxtaposed internally. The terminal and internal repeats divide the genome into a unique long (U_L) and a unique short region (U_S). Both unique components can invert relative to each other and, therefore, form 4 isomers which are found in equimolar populations in DNA extracted from virions.

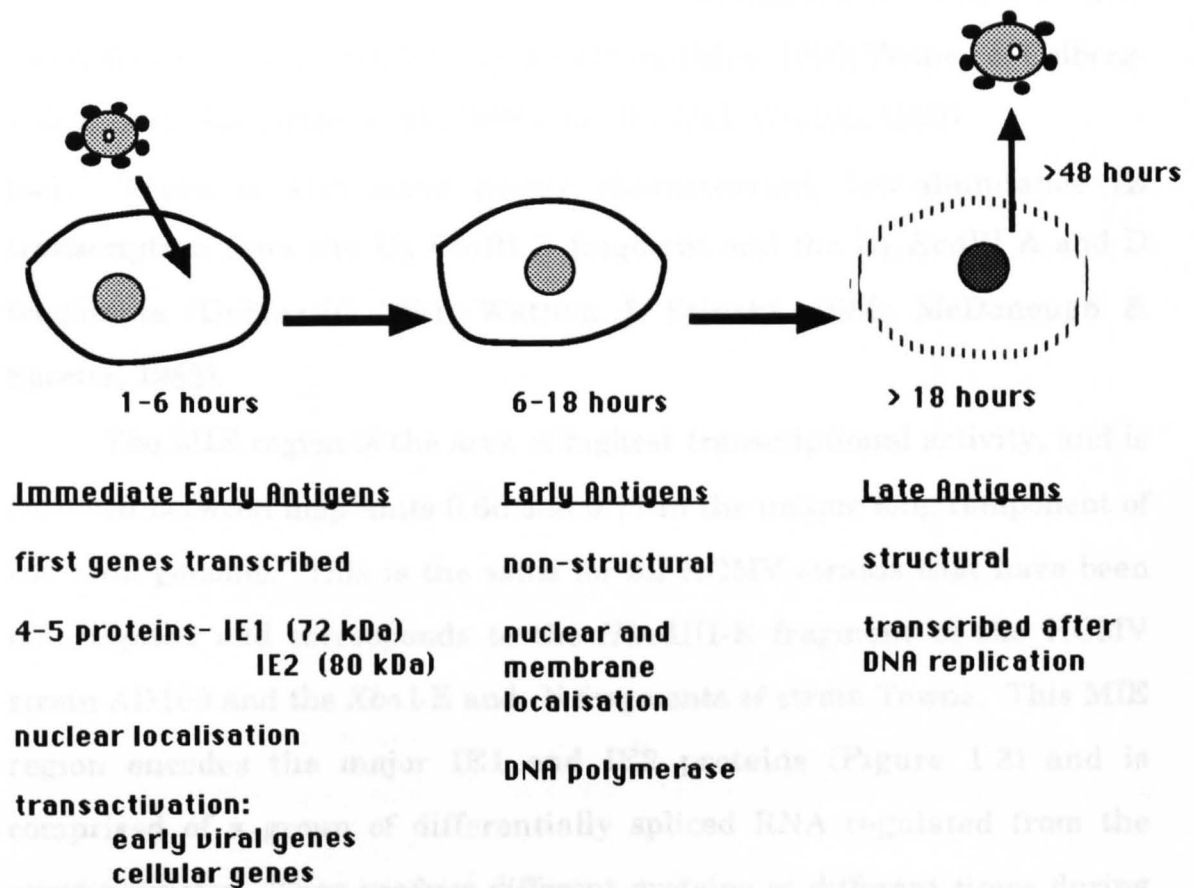


Figure 1.2. Gene Expression After In Vitro HCMV Infection of Permissive Cells. The diagram represents the 3 components of the viral cascade after entry of virus into a permissive cell. Immediate early genes are expressed first, followed by early and then late genes. The final event in productive infection is lysis of the cell and release of intact virions.

al., 1982). These are the major immediate early or MIE (Stinski *et al.*, 1983; Jahn *et al.*, 1984; Stenberg *et al.*, 1984; Akrigg *et al.*, 1985), the U_L36-38 (Wilkinson *et al.*, 1984; Tenney & Colberg-Poley, 1990; Tenney & Colberg-Poley, 1991; Kouzarides *et al.*, 1988), and the U_S3 (Weston, 1988)

loci. There is also some poorly characterized, low-abundance IE transcription from the U_S *Eco*RI B fragment and the U_L *Eco*RI A and D fragments (DeMarchi 1981; Wathen & Stinski, 1982; McDonough & Spector, 1983).

The MIE region is the area of highest transcriptional activity, and is localized between map units 0.66 and 0.77 in the unique long component of the viral genome. This is the same for all HCMV strains that have been investigated and corresponds to the *Hind*III-E fragment of the HCMV strain AD169 and the *Xba*I-E and -N fragments of strain Towne. This MIE region encodes the major IE1 and IE2 proteins (Figure 1.3) and is comprised of a group of differentially spliced RNA regulated from the same promoter. They produce different proteins at different times during lytic infection (Stenberg *et al.*, 1984; Akrigg *et al.*, 1985; Stenberg *et al.*, 1985; Stenberg *et al.*, 1989) and have been shown to exert their *trans*activational effects via different mechanisms (Hagemeier *et al.*, 1992).

IE1 is the most abundant polypeptide at IE times and first appears in the cytoplasm, within 1 hour following infection, where it is phosphorylated and transported to the nucleus. As mentioned above, IE1 is known to upregulate other cellular promoters and also has been reported to upregulate its own promoter in primary fibroblasts (Cherrington & Mocarski, 1989; Stenberg *et al.*, 1990). The latter activation occurs primarily via the 18 bp repeat elements present in the MIE enhancer/promoter (Cherrington & Mocarski, 1989) which are known

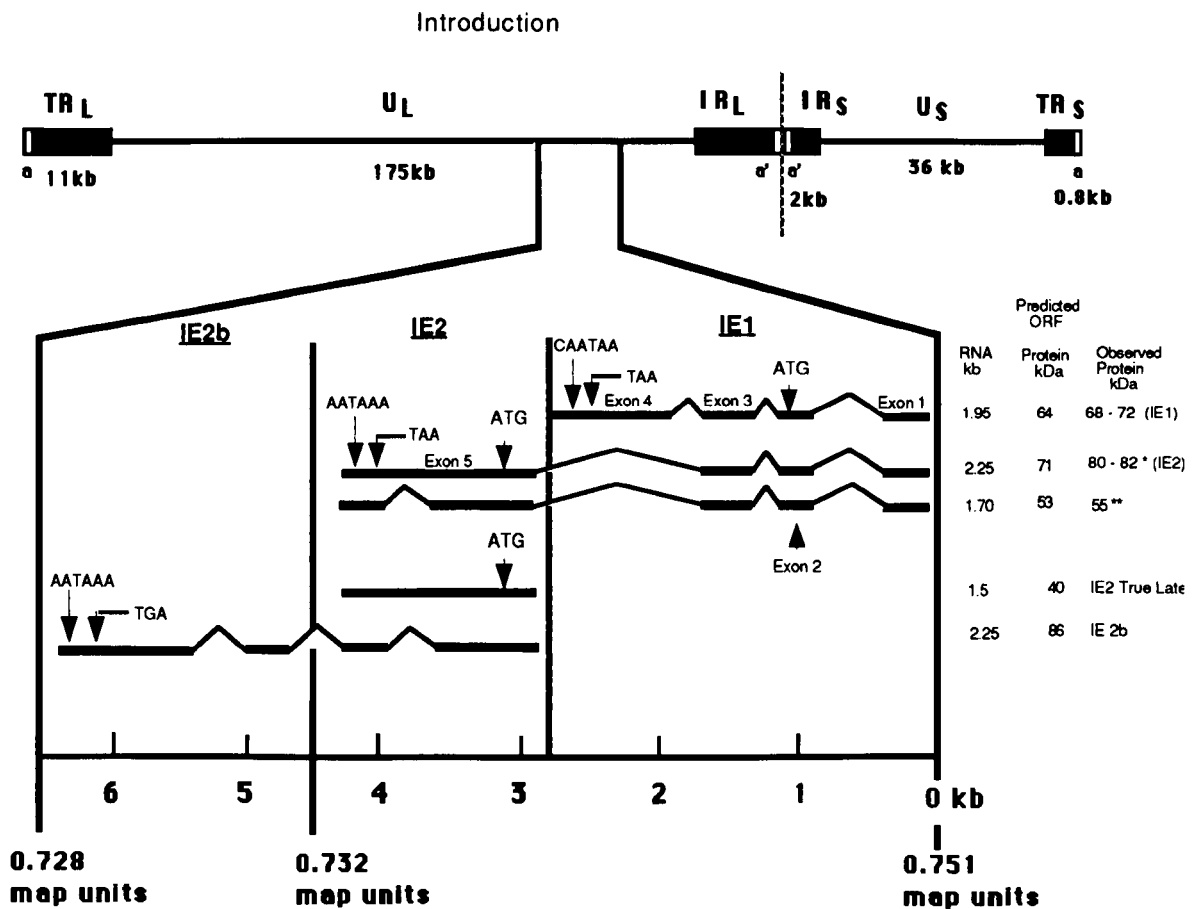


Figure 1.3. MIE Transcription Unit. IE1 and IE2 are transcribed in a right to left direction in the prototype genome arrangement (top) where terminal and internal repeats are labeled TR and IR and long and short components of the genome are labeled L and S, respectively (Figure 1.1). Predicted transcripts are shown as exons (thick lines) linked by spliced introns (thin lines). Transcriptional polyadenylation endonuclease cleavage sites (AATAAA), translational starts (ATG) or stops (TAA, TGA) are also noted. The IE1 and IE2 transcripts have the same 5' leader sequence generated from exons 1,2, and 3 alternately spliced to exon 4 or 5 to produce either IE1 or IE2, respectively. The single asterisk defines the IE2 transcript which is the functionally active transactivating and autoregulating IE2 protein (Pizzorno *et al.*, 1991), whilst the double asterisk defines the transcript which may be a cyclohexamide artifact (Pizzorno *et al.*, 1991). At late times of infection, a 1.5 kb transcript has been identified initiating from a cryptic promoter near the 5' end of exon 5. The IE2b transcript (Stenberg *et al.*, 1985) has been predicted from northern blots of infected permissive cells in the presence of cyclohexamide. The IE2b transcript has a functional TATA motif and CCAAT box just 3' to the IE1 gene and is a low abundance spliced transcript but is otherwise poorly characterized.

to contain a consensus sequence for the transcription factor NF- κ B (see below). Subsequently, Sambucetti *et al.* (1989) reported that a transcription factor, with all the characteristics of NF- κ B, played a central role in MIE enhancer/promoter activation in infected fibroblasts and was mediated through the IE1 protein. On the other hand, IE2 has also been shown to upregulate expression from heterologous HCMV early genes (Malone *et al.*, 1990) as well as heterologous non-HCMV promoters (Hermiston *et al.*, 1987) but is reported to down-regulate its own MIE enhancer/promoter (Pizzorno *et al.*, 1988). However, whilst no DNA-binding of IE1 to the MIE enhancer/promoter has been shown (Stenberg and Stinski, 1985), IE2 has recently been shown to directly bind to the cis repression signal (CRS) located between the TATA box and the cap site of the MIE enhancer/promoter (Lang & Stamminger, 1993).

The MIE RNA are controlled by a complex upstream enhancer-containing promoter (Stinski, 1990) that contains 4 functional units, a modulator region which is most 5' to the transcriptional start site, an NF1 cluster, an enhancer and a promoter (Figure 1.4). Each component of the MIE upstream regulatory region will be discussed individually, but I will use the term MIE enhancer/promoter to refer specifically to the intact MIE upstream regulatory region including the modulator, the NF1 cluster, the enhancer and the promoter.

By definition, initial expression from the MIE transcription unit occurs without de novo protein synthesis and must depend on viral and cellular factors. Expression may be mediated through a tegument protein component of the virion particle, possibly the phosphorylated 71 kd protein transcribed from the UL 82 gene, (Liu & Stinski, 1992) similar to activation of the HSV IE promoter by the viral tegument protein VP16 (O'Hare & Goding, 1988). Or, this expression may be mediated through attachment of virion components to the cell plasma membrane and activation of the

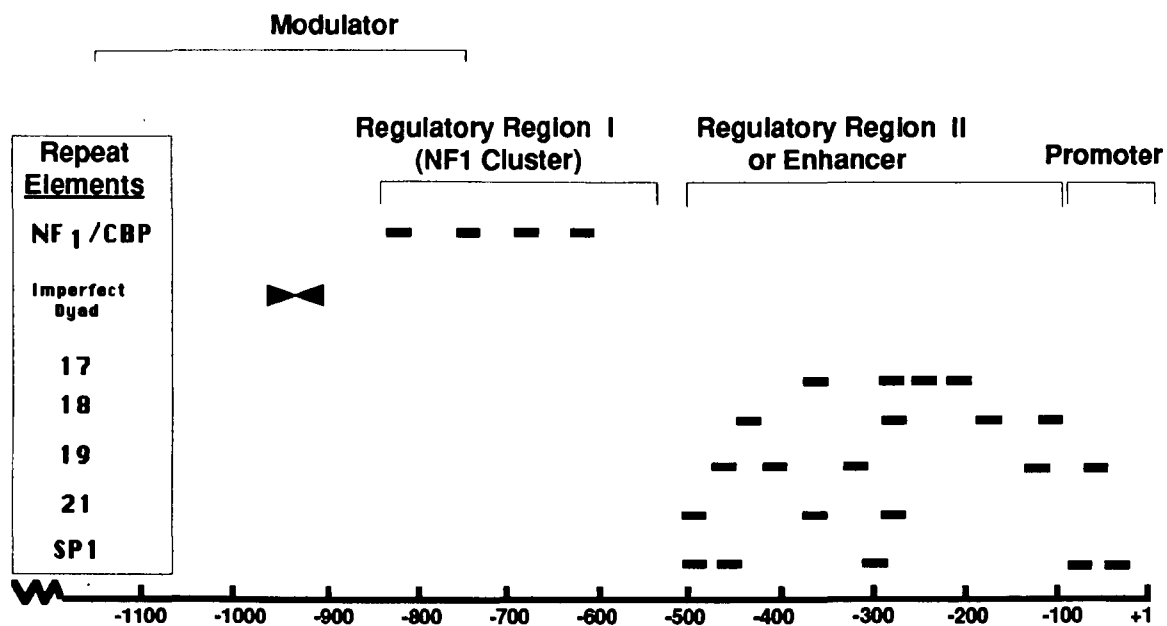


Figure 1.4. Upstream Enhancer-Containing Promoter of the MIE Transcription Unit. The repeats are highly conserved among human, simian, and murine CMVs and are separated by irregular stretches of nonrepetitive DNA. The promoter (-65 to +3) is sufficient for a low level of transcription and contains a CAAT box and a TATA element. The enhancer or regulatory region II (-520 to -65) stimulates downstream gene expression from cognate and foreign promoters and is one of the strongest known enhancer/promoters. The 17 bp, 18 bp, 19 bp, and 21 bp sequences present in regulatory region II are as follows, respectively: 5' ACTTGGCAGTACATCAA 3', 5' CTAAACGGGACTTTCCAA 3', 5' CCCATTGACGTCAATGGG 3', and 5' ACGGTAAATGGCCCG CCTGGC 3'. The modulator region (Lubon *et al.*, 1989; Shelbourn *et al.*, 1989b; Stamminger and Fleckenstein, 1990) which is -1145 to -750 has been found to act on promoter expression in a differentiation-dependent fashion. The imperfect dyad symmetry detailed in Shelbourn *et al.* (1989b), is shown in the modulator region upstream of regulatory region I. The sequence 5' TGGC\AN₅GCCAA 3' constitutes the NF1 sites present in regulatory region I. One NF1 site, not depicted here, is also present in the first intron of the IE1 gene.

proto-oncogenes *c-fos* and *c-jun* (Boldogh *et al.*, 1990). The modulator region (Nelson *et al.*, 1990) seen in Figure 1.4 has been shown to contain inducible DNaseI sites (Nelson & Groudine, 1986). In addition, this region has been shown to negatively regulate expression of the MIE enhancer/promoter in T cell lines and epithelial cell lines (Lubon *et al.*, 1989). And this region has been implicated in negative regulation in undifferentiated teratocarcinoma T2 cells, mediated at least in part, through binding of negative regulatory factors to an imperfect dyad symmetry located between -985 and -935 (Shelbourn *et al.*, 1989b). The functional significance of the NF1 sites is unknown. But, they do not appear to be involved in mediating negative regulation of the MIE enhancer/promoter in undifferentiated teratocarcinoma cells (Shelbourn *et al.*, 1989b). The enhancer contains multiple partially conserved repeats of 17, 18, 19, and 21 bp, as well as SP1 sites (Figure 1.4). These regions of the enhancer were shown to coincide with constitutive DNaseI hypersensitive sites (Nelson and Groudine, 1986) suggesting an open chromatin structure and that these areas may be active or potentially activatable. Some of the upstream regulatory regions and some of the enhancer repeats contain consensus sequences for known eukaryotic DNA binding proteins, e.g. the NF1 sites are putative sites for CAAT binding protein (CBP) super family, while the 19 and 18 bp elements have consensus sequences for cyclic AMP response element binding factor (CREB/ATF) and NF- κ B, respectively. The 17 bp elements can be deleted without negative effect on the strength of the promoter while the 19 and 18 bp repeats are functionally important elements for basal promoter activity (Fickenscher *et al.*, 1989). The 19 bp repeats have been shown to be necessary for cAMP responsiveness (Stamminger *et al.*, 1990). In addition, the 19 bp repeats have been noted to have some homology with the consensus sequence recognized by the transcription factor AP-1. AP-1 can be induced by the phorbol ester tetradecanoylphorbolacetate (TPA) which is thought to act through the protein kinase C pathway. Although phorbol esters have been implicated to mediate activation of the HCMV MIE

enhancer/promoter through induction of AP-1 in monocytic cell lines (Lebkowski *et al.*, 1987), upregulation could also have been due to removal of negative regulatory cellular factors upon phorbol ester differentiation. It is not clear that the putative AP-1 binding elements present in the 19 bp repeats play any role in direct AP-1-mediated activation of the MIE enhancer/promoter. The 18 bp repeat has been shown to be necessary for IE1 mediated transactivation of the MIE enhancer/promoter (Cherrington & Mocarski, 1989) and the factor which binds to this element has been shown to be indistinguishable from NF- κ B (Sambucetti *et al.*, 1989). As IE1 has not been shown to bind directly to DNA, it is possible that it acts through induction of NF- κ B (Sambucetti *et al.*, 1989) but exactly how this induction occurs is unclear. The 21 bp elements (Figure 1.4) have been shown to bind negative regulators of gene expression in undifferentiated teratocarcinoma cells (Kothari *et al.*, 1991) and also act in association with the imperfect dyad (Figure 1.4) to negatively regulate HCMV gene expression in an undifferentiated monocytic cell line, THP-1 (Sinclair *et al.*, 1992). Finally, the promoter (Figure 1.4) contains a TATA motif, located within 65 bp upstream of the transcription start site, which directs transcription using the cellular RNA polymerase II and other basal transcription factors.

Another region of the genome transcribed during IE time is located in the US, and has been designated the open reading frame HQLF-1 or *Hind*III fragment Q left reading frame (Weston & Barrell, 1986; Weston, 1988). Specifically, it is located in the *Eco*RI fragment L of strain AD169 and codes for 4 differentially splice RNA with common 5' and 3' ends. Although not as strong as the MIE enhancer/promoter, this gene also has an upstream enhancer with 11 bp and 18 bp repeats. The latter are homologous to those in the major IE enhancer/promoter but their significance is not well defined.

A potential glycoprotein has been described that is transcribed during IE times (Kouzarides *et al.*, 1988). It lies in the U_L between 0.21 and 0.24 map units and is located at the *Hind*III Z and J fragment junction. The 3.4 kb RNA codes for a predicted protein of 56 kd with characteristics of a membrane bound glycoprotein. It is unusual in the fact that no other known herpesviruses code for IE glycoproteins and there is no homology to known HSV glycoprotein genes. The protein product has not as yet, been identified .

1.3.2 Early and Late Genes

Early genes are defined as those genes which are able to be transcribed in the presence of inhibitors of viral DNA synthesis (Figure 1.2). In HCMV, early gene transcription can be detected throughout most of the genome, but most abundantly from the genomic repeat sequences and sequences adjacent to the repeats. The early phase of gene transcription extends for 24 hours or more and many early genes continue to be transcribed at late times. The most abundantly transcribed gene at early times is located in the large repeat sequences between 0.011 and 0.032 map units (Greenaway & Wilkinson, 1987). It is therefore present in 2 copies in every HCMV genome. This 2.7 kb unspliced RNA is transcribed 8-24 hours after infection and continues to be transcribed throughout infection, however a viral protein product has not been identified. One other early gene of importance is the DNA polymerase gene which is located between 0.31 and 0.33 map units. This RNA transcript is an unspliced 4.7 kb mRNA that encodes the viral specific 140 kd polymerase (Kouzarides *et al.*, 1987a).

Late genes are classically defined as those transcribed after DNA replication (Figure 1.2). However, for HCMV there are few genes which are true late genes. In fact, most late genes do not require viral DNA

synthesis for transcription and thus are often termed delayed early genes. Delayed early and/or late genes code primarily for structural components of the virion and include genes coding for the matrix-tegment, the nucleocapsid, and the glycoprotein components of the envelope. One true late gene used in this study was the matrix-tegment gene located at 0.63 to 0.65 map units in the UL coding for an unspliced 1.3 kb transcript. The product of this gene is a phosphorylated 28 kd protein which is extremely hydrophilic and highly immunogenic (Meyer *et al.*, 1988) and was used in this study in the examination of reactivation of HCMV from differentiated monocyte-derived macrophages.

1.4 CLINICAL ASPECTS OF HCMV INFECTION

1.4.1 Epidemiology

HCMV has become a successful pathogen of man due to primarily three factors. First, most acute infections in healthy subjects are asymptomatic and associated with little or no morbidity, therefore, allowing these individuals to continue normal activity at a time when viral shedding would be maximum. Second, the virus can be transmitted vertically and horizontally during either primary infection, reinfection, or reactivation. Thirdly, in the healthy individual, it is never eradicated but persists in the host for the rest of the life of the individual (Ho, 1990). Therefore, the seropositive individual is a ready source of virus which can be transmitted to susceptible individuals through an, as yet ill-defined, ability to reactivate. Reactivated virus can be transmitted through close sexual and nonsexual contact (e.g. passage through an infected birth canal, breast feeding from a seropositive mother, or close family or play group contact) or through organ transplantation and blood transfusion.

Classically, seroprevalence to HCMV ranges from 40-100% with a rough correlation between prevalence of antibody and the socioeconomic

conditions of the population (Table 1.1). A more recent epidemiological survey using the polymerase chain reaction technique suggested that the prevalence may be greater than previously reported (Bevan *et al.*, 1991). In the latter report the overall prevalence was 20% higher than data generated by latex agglutination on the same healthy blood donor population.

Age related seroprevalence studies suggest two primary periods associated with HCMV acquisition: the perinatal period and the age of sexual maturity. The latter is accounted for by both heterosexual and homosexual transmission (Drew *et al.*, 1981; Mintz *et al.*, 1983; Chandler *et al.*, 1985; Handsfield *et al.*, 1985; Collier *et al.*, 1987).

1.4.2 Pathogenesis

The pathogenesis of HCMV infections appears to be multifactorial involving both direct cytopathic effects of the virus and perhaps immunopathologic effects contributed by the host cell-mediated immune response (Grundy, 1990; Pasternack *et al.*, 1990). It is known that HCMV can produce tissue destruction in tissue culture and in vivo as manifested by cytomegalic cells. It is also known that decreasing viral load with the use of antiviral drugs correlates with improvement in some patients and can be used prophylactically to decrease the incidence of disease in other patients (Merigan *et al.*, 1992). The virus replicates so slowly in vitro and most likely in vivo (Alford & Britt, 1990) that immunocompetent hosts are often able to contain viral spread, therefore limiting cytopathology, whereas subjects with impaired immune function or the immature fetus manifest various levels of disease severity.

The immune response is generally considered to be protective. Antiviral antibodies are known to ameliorate disease in some cases through prophylaxis or therapy (Weimar *et al.*, 1990; Skarp-Örberg, 1990;

Snydman *et al.*, 1987; Winston *et al.*, 1987) and in general, the seropositive transplant recipient is not at as great a risk of serious HCMV disease as the seronegative recipient of a seropositive organ (Rubin, 1990). In addition, there is evidence to support the fact that cell-mediated immunity is protective in animal models and in the clinical setting (Starr & Allison, 1977; Quinnan *et al.*, 1982; Rook *et al.*, 1984; Ho, 1980; Reddehase *et al.*, 1985). Advances in defining the antigen targets recognized by CMV-specific cytotoxic T lymphocytes (CTLs) have shown that not only late antigens but also nonstructural HCMV IE and early antigens are recognized by HCMV specific CTLs in both murine and human systems (Reddehase *et al.*, 1984a; Reddehase *et al.*, 1984b; Reddehase *et al.*, 1986; Koszinowski *et al.*, 1987; Borysiewicz *et al.*, 1988a; Borysiewicz *et al.*, 1988b). One mechanism, therefore, by which CTLs contain viral infection may be lysis of cells at an early stage of infection preventing dissemination. Similarly, IE specific CTLs may be one mechanism by which the immune system controls persistent or reactivated latent virus in the normal immune carrier.

However, there are other reports, that indicate viral reactivation can occur even in the presence of high titer neutralizing antibody (Chou, 1990b). For example, prior immunity does not completely protect the seropositive allograft recipient. These patients are still at risk of reactivation of HCMV or of superinfection from HCMV strains carried in transplanted organs (Chou, 1990; Rubin, 1990; Smyth *et al.*, 1991). Similarly, severe manifestations of congenital HCMV infection in neonates born to HCMV immune mothers have been reported (Stagno *et al.*, 1973), although this is very rare.

In addition, both animal models and clinical evidence suggest that immunopathologic mechanisms of disease mediated by CTLs may exist (Shanley *et al.*, 1982; Grundy *et al.*, 1987a; Grundy *et al.*, 1990). There are

Table 1. Prevalence of Antibody to HCMV in Adult Populations

Location	Percentage with CF* antibody to CMV
Lyon, France	40
Freiburg, Germany	42
St. Gallen, Switzerland	45
Albany	45
Melbourne	54
Houston	79
Buenos Aires	81
Hong Kong	94
Sendai, Japan	96
Manila	100
Morocco, Morocco	98
Entebbe, Uganda	100

 Data from Ho, 1990.

* CF = Complement Fixation

many factors to be considered in evaluating the relevance of these reports (Pasternack *et al.*, 1990) but in general the lack of correlation of virus titers with severity of histopathology or with mortality (Grundy *et al.*, 1987a) make it necessary to consider immune mechanisms as another possible component of pathogenesis of disease.

Finally, aspects of HCMV's ability to interact with the host immune response should be mentioned. It has been reported that HCMV impairs the ability of lymphocytes and monocytes to produce and respond to interleukin-1 and interleukin-2 (Kapasi & Rice, 1988). As well, HCMV has been reported to impair function of HCMV-specific CTLs (Schrier and Oldstone, 1986) and to change the ratio of helper/suppressor T cells by decreasing the helper subset and increasing the suppressor subset (Carney *et al.*, 1981). In congenitally infected neonates, the same impairment of HCMV-specific CTLs has been demonstrated and a termination in viral shedding is correlated with a return of these cells to normal function.

1.4.3 Congenital HCMV

HCMV rarely causes disease in immunocompetent children infected in the perinatal period. Even infants infected in the immediate postnatal period either in the course of delivery or by postnatal breast feeding are rarely symptomatic. Unfortunately, this is not true in congenitally infected infants.

HCMV is the most frequent cause of congenital viral infections in humans (Weller, 1971) and is endemic worldwide leading to an average incidence of congenital infection in 1% of all newborn infants (Alford *et al.*, 1990). There is, in contrast to other viral diseases, a direct relationship between positive serostatus of the mother and prevalence of congenital infection. Maternal immunity, therefore, is unable to prevent both HCMV

reactivation during pregnancy and HCMV transmission to the fetus (Stagno *et al.*, 1977; Schopfer *et al.*, 1978; Stagno *et al.*, 1982). It has been shown that transmission of reactivated persistent virus is the most common source of congenital infection due to HCMV (Stagno *et al.*, 1977; Stagno *et al.*, 1982).

Historically, congenital HCMV infection, as defined by excretion of virus within the first week of life, is a condition that has been associated with severe signs and symptoms (Weller & Hanshaw, 1964). The most devastating disease complex, cytomegalic inclusion disease or CID (Weller & Hanshaw, 1964), has been described since the early parts of this century and represents about 10% of congenital infection due to HCMV. This severe form of congenital infection is overwhelmingly associated with primary infection of the mother and tends to be associated with infection in the early trimesters of pregnancy. Yet, not all fetuses born to mothers with primary infection during pregnancy will be affected and it has been described that the risk of severe disease may be predicted by positive amniotic fluid cultures (Grose *et al.*, 1992). It has recently been appreciated that a significant number of other neonates, who are asymptomatic at birth, are also at risk for serious sequelae. These infants, born to HCMV carriers, represent 90% of congenitally infected neonates and have no apparent manifestations at birth (Pass *et al.*, 1980).

Children born to HCMV carriers, who transmit infection from endogenous reactivated virus, have a high risk of developmental and other physical abnormalities (5-15%). These include: sensorineural hearing loss, microcephaly, motor defects (spastic diplegia or quadraplegia), mental retardation, chorioretinitis, and dental defects. These are usually recognized within the first 2 years of life, but progressive hearing loss can occur (Melish & Hanshaw, 1973; Saigal *et al.*, 1982). In the USA, reactivated virus in healthy female carriers who are pregnant leads to the

infection of 30,000-40,000 children per year of whom 4500-6000 will have an associated serious outcome as a direct effect of this infection (Yow, 1989).

1.4.4 Immunocompromised Adults and HCMV Infection

Analogous to congenital infections, reactivation of persistent virus either from transplanted organs, transfused blood, or the subjects own endogenous virus, is the most frequent source of infection in immunocompromised adults (Rubin, 1990). Primary infection is rare. HCMV is the single most important infectious agent affecting recipients of organ transplants, where approximately 2/3 of recipients will have HCMV infection 1-4 months after transplant. The high incidence of HCMV infections in these patients is due to a large reservoir of persistent virus (table 1.1) in the population in general, and is specifically correlated with the use of immunosuppressive drugs and allograft rejection (Rubin, 1990). In particular, the use of antithymocyte globulin is associated with the highest morbidity due to HCMV infection following transplantation.

1.5 HERPES VIRUS PERSISTENCE

All human Herpesviruses have the ability to persist after symptomatic or asymptomatic primary infection (Whitley, 1990; Miller, 1990a; Alford & Britt, 1990; Gelb, 1990; Pellett *et al.*, 1992). In general, these viruses have developed different mechanisms of persistence after primary infection and mechanisms to avoid elimination by the host immune system. Since these forms of chronic infection or carrier states are generally asymptomatic and are often associated with limited viral production they have been loosely termed latent infections. However, on a molecular level the term latency will be used here to mean: the reversibly nonproductive infection of a cell with or without limited viral gene expression by a replication-competent virus (see Garcia-Blanco & Cullen,

1991 for review). In addition, the word persistence has been used to mean continuous low level production of progeny virus in asymptomatic carriers (see Garcia-Blanco & Cullen, 1991 for review) but more generally to mean any mechanistically-based continuance of virus (Sissons *et al.*, 1991). Because it is convenient to have a general term which can be applied to viral maintenance in the normal human carrier when little is known about the actual mechanisms of maintenance, I will use the term persistence as an undefined mechanism of viral continuance. Virus persisting by production of progeny virus in the otherwise asymptomatic host will be termed low level lytic/replicative viral persistence.

The latent forms of the neurotropic Alpha subfamily viruses, human herpes simplex virus 1 and 2 (HSV1, HSV2) and varicella-zoster virus (VZV), are present in sensory ganglia (Baichwal & Sugden, 1988; Whitley, 1990; Alford & Britt, 1990) and show restricted gene expression. They can be reactivated in vivo to cause local lesions in the distribution of the sensory ganglia in which they reside (e.g. thoracic dermatomal distribution of zoster latent in thoracic dorsal root ganglion, or vermilion border lesions from HSV latent in the trigeminal ganglia). The mechanism of herpes simplex latency has been studied extensively. On a molecular level, HSV 1 appears to be present in an unintegrated episomal form with estimates of 20 copies per latently infected cell (see Stevens, 1989 for review). Latent HSV 1 and HSV 2 express RNA which maps to the long terminal repeat and are transcribed from the strand opposite the immediate early gene ICP0. The unspliced and spliced products of the latent transcript, 2 of which predominate, are unusual in the fact that they are not polyadenylated (although some less abundant polyadenylated transcripts do exist in the latent state in ganglia), they are uncapped, restricted to the nucleus and have no identified protein product (Wagner *et al.*, 1988; Stevens, 1989). It has therefore been theorized that these latency

associated transcripts or LATs (Stevens *et al.*, 1987) are nothing more than stable introns spliced from of an 8.3 kb pre-messenger RNA which has been found in lytic infection (see Garcia-Blanco & Cullen for review). However, the mature transcript from this 8.3 kb RNA is found only in lytic infection and not in latent infection and it would seem that the LATs represent more than just stable introns as they are found in both HSV1, HSV2 and in other neurotropic herpes viruses (Rock *et al.*, 1986; Cheung, 1989). As the LATs are antisense to and overlap one-third of the 3' end of ICP0, it has been postulated that they could form stable RNA duplexes with ICP0 transcripts as a mechanism of latency. However, LAT(-) mutants are not impaired in their ability to establish or maintain latency (Hill *et al.*, 1990; Block *et al.*, 1990) and LAT appears to function primarily in the reactivation process (Hill *et al.*, 1990). HSV 1 can be reactivated from latently infected ganglia *in vitro* by explantation followed by *in vitro* culturing, and *in vivo* by a number of stimuli (Whitley, 1990). Explantation has been shown to up regulate host cell expression of AP-1 and Oct 1, the latter of which is necessary for activation of immediate early gene expression by the tegument protein VP16 during lytic infection (O'Hare & Goding, 1988). The observation that only low levels of OCT 1 are found in ganglion cells have, therefore, been postulated as one mechanism of preventing HSV alpha gene expression during latency but other workers have postulated that an inhibitory protein (the neuronal form of OCT 2) can efficiently compete with VP16 for binding to viral DNA TAATGARAT motif in IE gene promoters and therefore repress expression (see Garcia-Blanco & Cullen, 1991 for review). The principle mediator, however, of the switch from latency to reactivation is likely to be ICP0. This gene product is sufficient and probably necessary for reactivation (Zhu *et al.*, 1990). It transactivates itself, as well as other immediate early genes, and early genes of the HSV cascade (see Garcia-Blanco & Cullen, 1991 for review).

For VZV, less is known. However, RNA expression has been documented in latently infected cells and explantation induces expression of IE RNA and some protein products, but without production of viral progeny as in HSV (see Stevens, 1989 for review). Unlike HSV LAT, these transcripts are found in at least 5 widely separated areas of the genome. It is not known in what physical state the genome exists in the ganglia. In addition, controversy exists as to which cell type harbors VZV genome during latency. Two groups have suggested that ganglionic neurons harbor VZV (Gilden *et al.*, 1986; Hyman *et al.*, 1983) while others (Croen *et al.*, 1988) have suggested that the virus is present in satellite cells of the ganglia. The latter group postulates that this location may account for the in vivo clinical differences between HSV reactivation (more frequent but less extensive) and VZV reactivation (much less frequent but more extensive lesions). It could be that satellite cells have to produce more virus to reach a threshold of infectious particles necessary to infect nearby neuronal cells. The larger production of viral particles leads to more extensive lesions upon reactivation, but the difficulty in reaching this threshold limits the number of clinical reactivations. Although these neurotropic viruses are related, the limited data on VZV latency suggest a different mechanism to HSV latency.

Epstein-Barr virus (EBV), the prototype human lymphotropic virus is a member of the Gamma subfamily and, exhibits both latency and low level replicative persistence depending on the cell type infected. It is latent in vivo in B cells with restricted gene expression, but there is some low level spontaneous reactivation (Miller, 1990a; Garcia-Blanco & Cullen, 1991). In epithelial cells, however, it is persistent with low level chronic production of progeny virus in the more terminally differentiated epithelial layers (see Allday & Crawford, 1988 for review; Young *et al.*, 1991). In B cells, the genome is maintained as an episome in

relatively high copy number, at approximately 60 copies per cell (see Stevens, 1989 for review). It is much less common to find it integrated (e.g. Namalwa cell line, 1B4 cell line). Most information concerning EBV latency is generated from studies of immortalized B lymphoblastoid cell lines (LCL), EBV-carrying Burkitt lymphoma (BL) cells, or nasopharyngeal carcinoma (NPC) cells. There are at least 6 nuclear antigens (EBNA 1-6) and 2 membrane antigens (LMP & TP) expressed in in vitro maintained LCL. In general, the transcriptional pattern is complex with long range splicing, spanning large areas of the genome (see Stevens, 1989 for review) and final mRNA products appear to be generated by alternative splice and polyadenylation site selections. The kinetics of EBNA protein expression suggest that EBNA2 and EBNA5 are expressed earlier than EBNA1 and EBNA3,4,6 (Allday *et al.*, 1989). In all three phenotypes of EBV carrying cells mentioned above, EBNA 1 is expressed (Klein, 1989) but the three cell types differ in their expression of the other 5 nuclear antigens and LMP. LCLs express all of the EBNAs and LMP while NPC express LMP only in addition to EBNA 1 and BL express only EBNA1 and none of the other latency associated genes. Three gene products, EBNA2, EBNA4 and LMP have been shown to contain epitopes for cytotoxic T cells and T-cell populations obtained from immunocompetent individuals readily kill autologous EBV-carrying LCLs in vitro (Moss *et al.*, 1978). Therefore, it is difficult to explain how LCL-like cells might survive in vivo. It may be that a more restricted pattern of expression than seen in LCLs may exist in vivo (Qu & Rowe, 1992). Activation of EBV, like HSV, can be stimulated by a number of physiologic changes. In vitro, phorbol esters such as TPA, antibody to immunoglobulin, butyrate and calcium ionophores have all been shown to increase the efficiency of reactivation. The principle mediator of such induction of the lytic cycle is the ZEBRA protein, expressed by the BZLF-1

open reading frame (Miller, 1990b). ZEBRA acts through AP-1-like DNA elements and shares protein sequence similarity with the *c-fos* component of AP-1 (Farrell *et al.*, 1989). In addition, TPA is thought to act through AP-1 sites which are present in the BZLF-1 promoter. Expression of ZEBRA is sufficient to trigger the viral lytic cascade (Flemington & Speck, 1990).

The Beta subfamily of human herpes viruses includes human herpes virus 6 (HHV6) and HCMV. HHV6, isolated by Salahuddin *et al.* (1986), has been shown to be the etiologic agent of roseola infantum (Yamanishi *et al.*, 1988) but its significance as a more serious pathogen is unknown. Viral replication can be induced by in vitro cultivation of T cells from patients with lymphoproliferative disorders or HIV and monocytes have been reported to be a site of persistence in healthy carriers (Kondo *et al.*, 1991). Beyond this little is known concerning the state of persistence.

Despite the fact that HCMV was initially isolated in tissue culture more than 30 years before HHV-6, the amount of information with respect to the state of persistence of HCMV is still limited. There are limited data regarding the cell type which harbors virus in the healthy carrier, even less is known about transcriptional expression during persistence and there is no information on the physical state of the genome in persistence. One report (Schrier *et al.*, 1985) suggested that lymphocytes harbored persistent HCMV in healthy carriers and expressed IE genes but productive infection was not detected by coculturing. However, the same group (Gnann *et al.*, 1988) was not able to find evidence of persistent virus in pre-transplant kidney biopsies using the same in situ technique and the same probes, despite the fact that lymphocytes would have been present. This may reflect the fact that in situ hybridization is not sensitive enough to detect rare copy persistent virus. Toorkey & Carrigan, 1989, have reported that many different cells in different organs harbor persistent

HCMV and express IE1 proteins but not late proteins. However, the tissues analyzed in this study were from autopsies and post mortem changes may influence viral IE expression and, therefore, not truly reflect the state of the virus in the healthy individual. Consequently, the sites persistence of HCMV are still poorly defined and there is no definitive evidence concerning transcriptional expression during persistence. Finally, there are no reports of successful reactivation of persistent HCMV from tissues of healthy carriers despite the fact that at least one group (Ibanez *et al.*, 1991) have attempted to reactivate HCMV from WBC of healthy carriers as the latter cells have been implicated to harbor transmissible virus (Yeager *et al.*, 1981; Adler, 1983; Tolpin *et al.*, 1985; Gilbert *et al.*, 1989, DeWitte, 1990).

1.6 OBJECTIVES OF THE STUDY

With the dearth of information about the state of persistence of HCMV in the healthy carrier, I chose to pursue this area of study with the following objectives in mind.

(i). Define specific cell types which harbor HCMV in the healthy carrier. As there is no animal model susceptible to human CMV and no in vitro latency system, it would be important to study a readily available tissue in the normal human host such that further molecular studies of persistence could be carried out more easily. As there is circumstantial evidence that HCMV exists in the peripheral blood of healthy carriers, most likely in the white cell fraction, I chose to determine the cell type(s) in the peripheral blood of healthy carriers that harbor HCMV. As persistent HCMV is likely present in very low copy number and as healthy volunteers would be able to donate only a limited amount of blood, this study would

require a highly sensitive system for analysis. Therefore, I decided to use the polymerase chain reaction as my primary methodology for detection of DNA since even a 50% efficiency of this method has a theoretical amplification power of over 10^9 with a nested PCR (Saiki *et al.*, 1985).

(ii). Identification of a specific site of persistence in the peripheral blood, would then permit an analysis of transcriptional activity of persistent HCMV in healthy carriers. Limited data suggest that there is a restriction in HCMV gene expression in PBMC during asymptomatic infection. However, the techniques used to define HCMV expression in these situations have been relatively insensitive. Therefore, reverse transcription coupled with PCR (RT-PCR) was chosen as a detection system for HCMV gene expression as it is highly sensitive. However, the need to use RT-PCR to detect low copy number transcripts would not permit a broad screen for evidence of HCMV transcription. Similarly, no animal or in vitro model of HCMV persistence/latency exists, in which to define putative latent/persistent transcription. Consequently, I decided to target specific HCMV genes of all phases of lytic infection.

(iii). Finally, once the spectrum of HCMV gene expression during persistence was defined, I would then analyze the mechanism of this regulation using peripheral blood cells and established myeloid cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1 SUBJECTS

All subjects were healthy adult volunteers.

2.2 SEROLOGY

2.2.1 ELISA

Serologic status was determined using a competitive ELISA system (CompEnz-CMV, Northumbria Biologicals), in which the ELISA plate was supplied, pre-coated, with HCMV antigen. Fifty μ l of serum and 50 μ l of horseradish peroxidase-conjugated murine Mab anti-HCMV were incubated overnight at 4 °C. After 4 washes with PBS-Tween, sample and control wells received a working strength of 3,3',5,5', tetramethyl benzidine substrate in an acetate/citrate buffer containing 0.025% (v/v) hydrogen peroxide, pH 6.0. This was allowed to incubate for 20 minutes at room temperature and the reaction was stopped with 2N H₂SO₄. Plates were read at 450 nm on an ELISA plate reader (Titertek Multiskan® MC, Flow) and average values were determined for each sample and for the control wells. All samples were prepared in duplicate along with 1 positive, 3 threshold, and 2 negative controls (supplied with kit). For duplicate test samples, obviously discordant values were disregarded and samples were re-tested. Results were interpreted as positive for any test serum when the A₄₅₀ was less than the mean A₄₅₀ of the threshold control serum (to be considered a valid control the mean of the threshold values had to be 35-65% of the mean value for the negative control). A negative result

occurred when the A450 of any test serum was greater than the mean absorbance value obtained in the threshold control serum.

2.2.2 Latex Agglutination

HCMV latex agglutination assay (CMVScan®, Becton Dickinson) was used to confirm all seronegative values derived from the HCMV ELISA as this assay will detect both IgM and IgG anti-HCMV antibodies. This commercially available assay was performed by placing 25 µl of test serum or control sera (supplied by the kit) onto individual circumscribed sample areas on a test card. Samples and controls then received one drop of latex particles which had previously been sensitized with CMV viral antigens. The cards were rotated in a moist chamber for eight minutes to mix the serum and latex particles. Readings were performed under bright light by comparing to controls. Visible clumping of particles indicated a positive result whereas, smooth and evenly dispersed particles indicated negative agglutination.

2.3 MAINTENANCE OF CELL LINES

2.3.1 THP-1 Cells

THP-1 cells (Tsuchiya *et al.*, 1980) were obtained from the ATCC. This immortalized human cell line consists of premonocytic human cells originating from a child with acute monocytic leukemia. Upon differentiation with a number of chemicals, these cells develop into macrophages (Tsuchiya *et al.*, 1982) despite the fact that they retain some granulocyte surface markers (Skubitz *et al.*, 1983). Cells were maintained as suspension cultures in RPMI medium with 10% FCS and were split 1 to 4 every 5-7 days.

2.3.2 F2002 and MRC5 Cells

F2002 and MRC5 (Jacobs *et al.*, 1970) fibroblasts were obtained from Flow and the ATCC, respectively. These cells were maintained in MEM Eagle medium (MEM) with 10% FCS. Cells were split 1 to 2 every 5-7 days. These cells undergo 42-46 population doublings before onset of decline in proliferation. For all experiments, passages ranging from 26-40 were used. To passage adherent monolayers, cells were washed twice in MEM without serum then detached with trypsin-EDTA (0.5g trypsin, 0.2g EDTA, 0.85 g NaCl/L, Gibco) and incubated at 37 °C for 5-10 minutes. Trypsinized cells were divided into new flasks and fresh MEM 10% FCS medium was added.

2.3.3 Media and Cryopreservation

RPMEI 1640 is an enriched formulation with a more extensive application for mammalian cells and was originally formulated for suspension cultures while MEM Eagle medium (Earle's salts) was designed for use with primary mammalian cell cultures e.g. MRC-5 cells. Both media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 1 g/L sodium bicarbonate. For freezing, THP-1 cells were pelleted and resuspended in FCS with 10% DMSO then frozen at -80 °C overnight then transferred to liquid nitrogen for long term storage. To freeze HELF cells, 1 confluent T150 flask was trypsinized resuspended in 10 ml of MEM 10% FCS and centrifuged 200 xg for 5 minutes. Pelleted cells were resuspended in 4 ml of FCS with 10% DMSO and divided into 4 cryogenic vials (Corning). Vials were placed at -80 °C overnight then transferred to liquid nitrogen for long term storage.

2.4 VIRUSES

2.4.1 HCMV AD169 Strain

HCMV strain AD169 was obtained originally from the ATCC (cat. no. VR-538), and was plaque purified twice. The virus was then propagated in F2002. Using a MOI of 0.01, the medium from HCMV infected fibroblasts was harvested on a daily basis until the cell monolayer was destroyed by CPE. Supernatants were clarified, aliquoted and frozen at -80 °C. Aliquots were plaque-titered using 10-fold dilutions of viral supernatant on HELF with a 1% agarose (SeaPlaque®, FMC Bioproducts) overlay.

2.4.2 HCMV Clinical Isolates

Clinical HCMV isolates, specifically the strain designated M, was a gift of Dr. Roz Smyth and Dr. Jane Minton and was cultured from a bronchoalveolar lavage sample from a heart-lung transplant patient.

2.5 ISOLATION OF PBMC & PMNL BY DENSITY-GRADIENT CENTRIFUGATION

Peripheral blood was obtained by percutaneous venepuncture and anticoagulated with either heparin (10 units per ml of blood) or with 0.5M EDTA pH 8.0 (final concentration 4 mM). Ten ml of anticoagulated blood was layered onto 10 ml of Lymphoprep™ (9.6% (w/v) Sodium metrizoate and 5.6% (w/v) polysaccharide, Nycomed Pharma AS) in a 25 ml universal tube (Sterilin, England) and centrifuged at 800 xg for 15 minutes with no brake upon deceleration. The density gradient (Bøyum, 1968) yielded an upper plasma layer, a cloudy white PBMC band at the plasma Lymphoprep™ interface and a PMNL-enriched fraction lying at the top of the aggregated RBC pellet as shown in Figure 2.1.

The PBMC and the PMNL-enriched fraction were both washed twice as follows: 10-15 ml of sterile PBS were added to the cells and the tube was vigorously mixed to break-up cell clumps. After centrifugation at 450 xg for 10 minutes; the cell pellet was washed for a final time by discarding the supernatant and resuspending in 5 ml of sterile PBS followed by centrifugation at 200 xg for 5 minutes. All steps were performed in a sterile manner with sterile disposable plastic pipettes in a PCR-safe laminar flow hood in which neither virus or infected tissues had been processed.

2.6 SEPARATION OF SPECIFIC CELL POPULATIONS

2.6.1 Isolation with Mab and FACS

PBMC were labeled with mouse monoclonal anti-human CD3 IgG (derived from the mouse hybridoma cell line OKT 3 CRL 8001, purchased from the European Collection of Animal Cell Cultures) and a fluorescein isothiocyanate-conjugated F(ab')₂ fragment of rabbit anti-mouse IgG (Dakopatts), or with fluorescein-conjugated mouse anti-human CD14 antibody (Becton Dickinson). All antibody incubations were carried out at room temperature for 1 hour, were washed twice in 10 ml of PBS after each incubation and fluorescein-conjugated antibody incubations were performed in the dark.

FACS was performed on a EPICS C flow cytometer where droplets were electrostatically separated into positive and negative populations (gated using forward and 90 degree light scatter) with single fluorescence.

2.6.2 Isolation of Monocytes by Adherence

Adherent cells were isolated from PBMC or depleted from PMNL by plating the respective cell populations on 14 cm plastic petri dishes in PBS and incubating for 1 hour at 37 °C in 5 % CO₂ (Treves et. al., 1980). PBMC

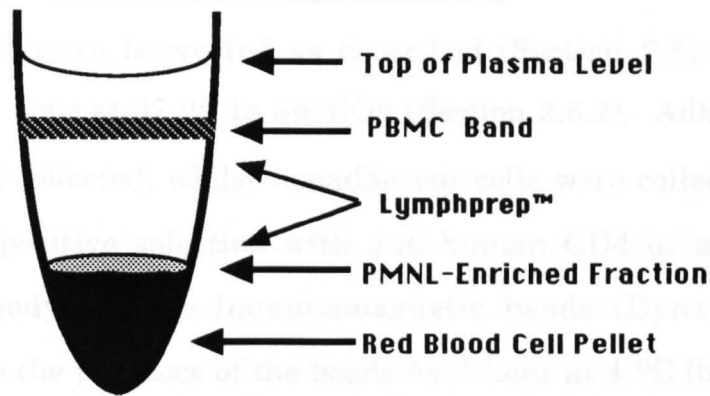


Figure 2.1. Density-Gradient Separation of Whole Blood. The figure shows a diagram of the separation achieved after density-gradient centrifugation with whole blood and Lymphoprep™ and abbreviated labels are detailed as follows: PBMC = peripheral blood mononuclear cells and PMNL = polymorphonuclear leukocytes .

2.6.4 Purity Analysis

To analyze purity after FACS, a small sample of cells was either resorted with the EPICS C flow cytometer or was fixed with paraformaldehyde and analyzed on the Coulter EPICS Profile machine.

In addition, adherent and non-adherent cells were stained with a fluorescein-isothiocyanate conjugated mouse anti-human CD14 or CD3 antibody to determine the percentage of mature monocytes or CD3 positive T lymphocytes present in cells adhering to plastic.

adherent cells, representing primarily monocytes, and PMNL-enriched non-adherent cells (neutrophils, eosinophils, and basophils) were collected.

2.6.3 Isolation with Immunomagnetic Beads

PBMC were harvested as described (Section 2.5) and adhered to plastic for 1 hour at 37 °C in 5% CO₂ (Section 2.6.2). Adherent cells were scraped and collected, whilst nonadherent cells were collected and divided in half for positive selection with anti-human CD4 or anti-human CD8 using antibody specific immunomagnetic beads (Dyna^l®). Cells were incubated in the presence of the beads for 1 hour at 4 °C (bead to cell ratio: 10-1). Beads with attached cells were washed 6 times in 10 ml of PBS per wash to remove any unbound cells. Finally, beads with attached cells, were resuspended in 2 ml of PBS. 200 µl were further processed with DetachaBead (Dyna^l®) according to manufacturer's directions and released cells were then cytopun (Cytospin 2, Shandon) onto glass slides for purity analysis. The remaining 1.8 ml of cells attached to beads were magnetically separated and the cell pellet was processed for DNA (section 2.7).

2.6.4 Purity Analysis

To analyze purity after FACS, a small sample of cells was either resorted with the EPICS C flow cytometer or was fixed with paraformaldehyde and analyzed on the Coulter EPICS Profile machine.

In addition, adherent and non-adherent cells were stained with a fluorescein-isothiocyanate conjugated mouse anti-human CD14 or CD3 antibody to determine the percentage of mature monocytes or CD3 positive T lymphocytes present in cells adhering to plastic.

In order to detect the presence of contaminating monocytes in non-adherent PMNL fractions, a staining technique to detect esterase using α -naphthyl butyrate was employed. Slides of non-adherent PMNL were air dried and fixed for 30 seconds with cold buffered formalin acetone (pH 6.6). After rinsing with tap water, slides were allowed to incubate for 45 minutes at room temperature with a substrate of α -naphthyl butyrate (Sigma) in phosphate buffer (pH 7.4) and a coupler of hexazotised new fuchsin (Sigma). Slides were then rinsed in tap water and counterstained with Mayers hematoxylin (Sigma) for 45 seconds. Resultant enzyme activity is seen as red-brown granules in monocytes.

Purity of CD8+ and CD4+ cells was analyzed after using DetachaBead and cytopspining cells onto slides. Cells were fixed on slides in acetone/methanol (1:1) for 3 minutes at -20 °C and air dried. Then, slides with CD8 or CD4 cells were stained with either CD8 or CD4 antibody, respectively, or CD4 or CD8 antibody, respectively, (CD4 and CD8 antibodies were obtained from the Royal Free Hospital School of Medicine, Academic Department of Immunology), for 1 hour at room temperature. Following 2 washes in PBS/0.1% (v/v) Tween 20, a 1/30 dilution of rabbit anti-mouse fluorescein isothiocyanate-conjugated F(ab')₂ fragment (Dakopatts) was applied to the cells for a further 60 minutes at room temperature. Following 2 more washes, slides were air dried and Citifluor was applied prior to UV microscopy. Positive controls were unselected non-adherent cells processed in the same way. In addition, cells were analyzed for the presence of monocytes by butyrate esterase staining as described above. All spots were performed in duplicate for each staining technique.

2.7 DNA EXTRACTION

2.7.1 RNase/Proteinase K Method

Cells were washed, pelleted, and resuspended in 250 μ l of TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA). An equal volume of 2x PK buffer (300 mM NaCl, 200 mM Tris-HCl pH 7.5, 25 mM EDTA pH 8.0) was added. One-tenth volume of 10% SDS (sodium dodecyl sulfate) was added and mixed well. RNase was added to a final concentration of 2 mg/ml and the solution was incubated at 37 °C for 1 hour. Following this, Proteinase K was added to a final concentration of 6 mg/ml and incubated at 37 °C for 2 hours. Samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged at 10,000 xg for 5 minutes. The aqueous phase was recovered and extracted with an equal volume of chloroform/isoamyl alcohol (24:1), and centrifuged at 10, 000 xg for 5 minutes. DNA was precipitated from the aqueous phase with 2.5 volumes of ethanol at -20 °C overnight. The precipitated DNA was pelleted at 10,000 xg for 15 minutes, washed once with 70% ethanol, dried and resuspended in TE. DNA concentrations were estimated by comparison to known concentration standards of DNA on an ethidium bromide-stained 0.6% agarose gel.

Some DNA samples were extracted using the RNase/Proteinase K method. These were the samples used for the CD3, CD14, and initial adherent cell studies (see Results Section 3.4.2). However, as this DNA preparation often led to a high percentage of non-amplifiable DNA samples (approximately 25%), an alternative method using sodium perchlorate was employed (section 2.7.2).

2.7.2 Sodium Perchlorate Method

DNA was extracted using a modified sodium perchlorate method (Weetman *et al.*, 1990). Pellets of 10^5 to 10^6 cells were thoroughly mixed

with 600 μ l of solution 1 (100 mM NaCl, 50 mM EDTA, pH 8), followed by 50 μ l of solution 2 (25% SDS), and finally 150 μ l of solution 3 (5M Na perchlorate). The cell pellet solution was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, Sigma), centrifuged at 10,000 g for 15 minutes at room temperature. The aqueous phase was extracted with chloroform:isoamyl alcohol (24:1), and precipitated with an equal volume of isopropanol for a minimum of 15 minutes on ice. The DNA was pelleted by centrifugation at 10,000 g for 15 minutes at room temperature, and washed twice with 70% ethanol. Ethanol was removed and the damp pellet was resuspended in TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA, Sigma). DNAs were dialyzed overnight at 4 $^{\circ}$ C against 1000-fold volume of TE. After dialysis, DNA concentration was analyzed by measuring the $A_{260\text{nm}}$ in a 1 cm path length of a diluted sample on a SP6-450 UV/VIS Spectrophotometer (PYE UNICAM). Final concentration of DNA was calculated as follows: $A_{260\text{nm}} \times 50$ (1 A_{260} unit = 50 μ g/ml) \times dilution factor = μ g/ml. In some cases where the DNA samples were extremely small, 1 μ l of DNA was run on an 0.6% agarose gel and the concentration was visually estimated against known standards. DNA was frozen at -20 $^{\circ}$ C for storage.

All DNA extractions were performed in a PCR-safe manner (section 2.10).

2.8 RNA EXTRACTION

2.8.1 Total RNA Extraction

A total RNA extraction kit (Promega) based on the method of Chomczynski *et al.* (1987), was used. All manipulations were carried out on ice and in a PCR-safe manner (see section 2.10).

Medium was removed from primary monocytes or MDM cells and any floating cells were pelleted at 300 xg for 5 minutes in PCR-safe sterile disposable 50 ml falcon tubes. Medium was decanted and pellets were held on ice until processed. Denaturing solution (DN solution), containing guanidinium thiocyanate in a citrate/sarcosine/ β -Mercaptoethanol buffer, was added directly to adherent monolayers and cells were scraped (using PCR-safe sterile disposable plastic cell scrapers, Greiner) into the DN solution. Adherent cell extracts were pooled from all flasks using PCR-safe sterile disposable pipettes and added to the cell pellets collected initially from cells floating in the medium. Extracts were acidified with 1/10 volume of 2M Na acetate, pH 4.0. An equal volume of acid phenol:chloroform:isoamyl alcohol was added, mixed well and allowed to settle for 15 minutes on ice then centrifuged at 10,000 xg for 30 minutes at 4 °C. The aqueous phase was recovered and precipitated with 100% isopropanol on dry ice for at least 30 minutes or overnight at -80 °C. RNA was pelleted by centrifuging at 10,000 xg for 30 minutes at 4 °C. Pellets were resuspended with 100 μ l of DN solution, pooled and reprecipitated with an equal volume of 100% isopropanol on dry ice for at least 30 minutes or overnight at -80 °C. RNA was pelleted at 10,000 xg for 30 minutes at 4 °C. The pellet was washed with 75% ethanol and centrifuged at 10,000 xg for 5 minutes at 4 °C. After removing all ethanol by aspiration, pellets were further processed as detailed below with RNase-free DNase (section 2.8.2).

2.8.2 DNA-free RNA

The acid/phenol-based RNA isolation did not yield DNA free RNA preparations (as determined by ethidium bromide stained agarose gel electrophoresis of RNA and by intron-spanning RT-PCR) and it was therefore necessary to remove contaminating DNA from all RNA isolated

from healthy subjects. Using RQ1™, an RNase-free DNase (Promega), RNA samples were processed as follows: After the 75% ethanol wash RNA pellets were resuspended in 500 µl of RQ1 Buffer (40 mM Tris-HCl pH 8.3, 10 mM NaCl, 6 mM MgCl₂, and 10 mM CaCl₂) with 2 U of rRNasin (Promega) and 15 U of RQ1™. The mixture was incubated at 37 °C for 15 minutes, then 1/2 volume of stop solution (50 mM EDTA, 1.5 M Na acetate, and 1% SDS) was added. RNA was extracted with acid phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 1 volume of isopropanol, pelleted at 10,000 xg for 30 minutes at 4 °C, and washed once with 75% ethanol. The ethanol was removed by aspiration and the RNA pellet was resuspended in RNase-free water (Promega). For long term storage, RNA was reprecipitated with Na acetate pH 4.0 (final 0.25 M), and 2.5 volumes of 100% ETOH and frozen at -70 °C.

There were no solutions provided with the RQ1™ enzyme, therefore, all chemicals were prepared in a PCR-safe manner, see section 2.10.

2.8.3 RNA Integrity Analysis and Concentration

RNA integrity was examined by analysis on denaturing agarose gels and RNA concentration was determined by measuring optical density at 260 nm after removing all background DNA.

Formaldehyde-denaturing agarose gel was prepared by melting 1.2 g of agarose (ultraPURE™ Agarose, Electrophoresis Grade, BRL) in 102 ml of H₂O. The agarose solution was cooled to 50 °C and then the following were added in a fume cupboard: 24 ml of 5x MOPS buffer (1x MOPS = 20 mM MOPS, 8 mM Na acetate, 1 mM EDTA; prepared in 0.1% DEPC treated water and brought to pH 7.0 with 2N NaOH) and 6.5 ml of 37% formaldehyde. The gel was cast immediately.

Five µl of the RNA sample was evaporated to dryness by spinning under vacuum for 10 minutes in a Savant Speed Vac SC 100 model.

Samples were resuspended in 20 μ l of loading buffer (50% (v/v) deionized formamide, 6.5% (v/v) formaldehyde, 1x MOPS, 50 mg/ml ethidium bromide and 0.02% bromophenol blue). Resuspended samples were denatured at 80 °C for 20-30 minutes, placed on ice for 5 minutes, and electrophoresed in a formaldehyde-denaturing gel submerged in 1x MOPS and run at 3-5V/cm for 1-1.5 hours. The electrophoresed RNA samples were examined on a transilluminator (UVP, Inc.) at 312 nm for the integrity of ribosomal RNA as well as for the presence of any DNA.

To determine RNA concentrations, 1 μ l of sample was diluted in 500 μ l of H₂O and the absorbance at 260 nm ($A_{260\text{nm}}$) was measured through a 1 cm path length. If gel electrophoresis showed no evidence of DNA, this absorbance was deemed to represent RNA and RNA concentration (μ g/ml) was determined as follows: $A_{260} \times 40$ ($1A_{260} = 40 \mu\text{g/ml}$ of RNA) \times dilution factor.

2.9 REVERSE TRANSCRIPTION

2.9.1 First Strand Synthesis

First strand reverse transcription was carried out using 10 μ g of total RNA (approximately equivalent to 10^5 cells) and is detailed in section 4.2.1.

2.9.2 RT Sensitivity Analysis

Total RNA was extracted from AD169 strain HCMV infected MRC5 cells, which had been infected for 1 week and showed 100% CPE. For every assay, 10 ng and 0.1 ng of RNA from HCMV infected cells, equivalent to 10^2 and 1 HCMV infected cell respectively, were reverse transcribed in order to assess sensitivity of the RT.

2.10 PREVENTION OF PCR CONTAMINATION

2.10.1 Sources of Contamination

Contamination is a well recognized problem with PCR (Kwok & Higuchi, 1989; Ivinson and Taylor, 1991; Jackson *et al.*, 1991; Clackson *et al.*, 1991) and particularly when using high cycle number or nested-PCR, which are necessary to detect the extremely low copy genes and gene transcripts often found with persistent viral genomes. Amplification products from either the primary or the secondary phase of a nested PCR can easily be passed into other solutions from contamination of micropipettes or through handling with contaminated gloved hands. This has been termed PCR product carryover contamination (Kwok & Higuchi, 1989; Williams, 1989; Gibbs *et al.*, 1989; Sarkar & Sommer, 1990). DNA and RNA preparations from HCMV-infected tissue culture represent another source of micropipette or handling contamination that may be "carried over" to PCR solutions.

Moreover, when PCR target DNA is also in use as plasmid clones in the same laboratory, other problems occur. For example, plasmid handling contaminates gloved hands which may then contaminate PCR reactions (Kwok & Higuchi, 1989). Similarly, plasmid DNA may become aerosolized (one possible mechanism is through autoclaving) and become an airborne source of contamination.

Rigorous procedures to minimize PCR contamination, as detailed in sections 2.10.2 and 2.10.3, were used in the preparation of all solutions for PCR and RT as well as for all solutions used in preparing DNA and RNA samples. When preparing positive controls from infected tissues, all work was done on a separate bench in a completely separate laboratory with different equipment and solutions.

2.10.2 Prevention of Carryover Contamination

Prevention of carryover contamination required that all pre-amplification PCR (pre-PCR) work be carried out in an area that was completely separate from any post-amplification PCR (post-PCR) work. This was accomplished by the use of a laminar flow hood that was dedicated to the preparation of PCR-safe solutions and DNA and RNA from uninfected tissues. In addition, all equipment used for pre-PCR work was dedicated to that purpose. Finally, to prevent micropipette contamination, all pipette tips used for pre-PCR work were sterile ART™ (aerosol resistant tips, Continental Laboratory Products).

For the secondary phase of the nested PCR, all reaction mixes were initially prepared in the PCR-safe hood. Then, target DNA from the first phase amplification was added outside the hood on an otherwise PCR-safe bench using ART™ tips with a special set of pipettes used only for secondary phase of PCR.

To prevent handling contamination, gloves were changed whenever entering the PCR-safe hood. All chemicals for PCR were dedicated to that purpose and kept in the PCR-safe lab. All equipment, including racks, tubes, microcentrifuge, etc. were also dedicated solely to pre-PCR work and kept in the PCR-safe lab. In addition, all PCR racks were rinsed in 0.25 M HCl prior to use in the PCR-safe hood (Kwok & Higuchi, 1989). Disposable sterile gowns, were routinely used while working in the PCR-safe hood (Bloch, 1992).

2.10.3 Prevention of Airborne Contamination

The laminar flow hood, dedicated to pre-PCR work, efficiently reduced airborne contamination of PCR reactions. Where possible, all chemicals were purchased as solutions. If this was not possible, chemicals were purchased solely for PCR and solutions prepared only

under PCR-safe conditions in the laminar flow hood. Chemicals were purchased in small quantities and made to solution by adding double-processed sterile water (Sigma) directly to the bottle contents.

Because the use of the laboratory autoclave gave rise to autoclaved solutions which were routinely positive for HCMV IE PCRs (probably due to contamination from autoclaved plasmid waste), the use of any autoclaved materials was avoided. All plastics were purchased sterile (if possible) and disposable. PCR tubes and 2 ml eppendorf tubes were used directly from the sealed bags and kept in the PCR-safe hood until used.

2.11 POLYMERASE CHAIN REACTIONS

2.11.1 PCR Oligonucleotide Primer and Probe Synthesis

PCR oligonucleotides were synthesized on the Applied Biosystems 381A synthesizer using β -cyanoethyl phosphoramidite chemistry. After synthesis, oligonucleotides were extracted from the column with 1 ml of ammonia by incubating for 1-2 hours at room temperature, and deprotected for 8-18 hours by further incubation at 55°C. Oligonucleotides were precipitated on dry ice for 10 minutes in an equal volume of KAW buffer (3M potassium and 5M acetate) and 3 volumes of 100% ETOH. The oligonucleotide precipitate was pelleted at 10,000 xg for 10 minutes. The pellet was washed in 80% ETOH and all ETOH was removed by aspiration, as opposed to dessication, prior to resuspending in 100 μ l of TE. One μ l of the stock primer was diluted in 1 ml of H₂O and an A_{260nm} was measured through a 1 cm path length. Oligonucleotide concentration was determined by the following equation: $A_{260nm} \times 20$ (1 A₂₆₀ unit = 20 μ g/ml of an oligonucleotide) \times dilution factor = μ g/ml. The stock solution was routinely stored at -70 °C until use.

The general strategy for choosing primer sequences (Taylor, 1991; Saiki, 1989) was:

1. Primers should be about 20 bp in length.
2. Primers should flank a product of small enough size to be easily visualized on agarose gel electrophoresis and subsequently easily transferable by Southern blotting. This size was approximately 250-500 bp.
3. Primers should have about 50% G-C and 50% A-T composition so that annealing temperatures will be 55 °C ($T_{\text{annealing}} = T_{\text{melting}} - 5 \text{ °C}$).
4. Primer sequences should not have palindromes (to avoid stable loop formation) or any complementarity especially 3' complementarity (to avoid primer-dimer amplification).
5. If possible primers should flank introns so that DNA and cDNA amplification products may be differentiated. This is helpful in distinguishing cDNA plasmid contamination of amplified DNA samples.

2.11.2 HCMV Immediate Early Gene PCRs

The HCMV Immediate Early Gene PCRs were used to amplify both DNA and cDNA products. The details of the specific PCRs, which include IE1, IE2 and US3 (HQLF-1) gene amplifications, are presented in the materials and methods sections of chapters 3 and 4 (see sections 3.2.2, 4.2.2, 4.2.3 and 4.2.4). All PCRs were nested for maximum sensitivity (Mullis & Faloona, 1987; Bell, 1989; Jackson *et al.*, 1991).

2.11.3 HCMV Early Gene PCR

For experiments in sections 3.4.4 and 3.4.5 (HCMV DNA studies in Lymphocytes, and Polymorphonuclear Cells of Healthy Carriers) the early gene PCR was used. Details of the PCR are presented in the pertinent results section (section 3.2.3).

2.11.4 HCMV Late Gene PCR

For RT-PCR experiments, a late gene PCR was used to assess the degree of reactivation. The gene chosen for this analysis was a major 28 kd structural phosphoprotein (pp28). Whilst, many late genes express RNA prior to true late times, this gene is an example of a late gene whose RNA is only expressed at late times of infection, after viral DNA replication (Meyer, et. al., 1988). Details of the PCR are presented in the pertinent results section 4.2.5.

2.11.5 Histidyl-tRNA Synthetase PCR

The histidyl-tRNA synthetase PCR was used as a control PCR for the ability to amplify DNA and cDNA after reverse transcription. As the amplification product spanned an intron, the cDNA product was 128 bp while the DNA product was approximately 370 bp (Tsui & Siminovitch, 1987; Corrochano, 1991). Histidyl-tRNA synthetase is a multicopy housekeeping gene and required analysis of only 10 ng of DNA and 2 µl of a 50 µl RT mix (the amount of total RNA from approximately 4×10^3 cells). The sequences of the primers and the intraexonic probe were:

Sense Primer: 5' > TCATCAGGACCCAGCTGTGC <3',

Anti-sense Primer: 5' > C TTCAGGGAGAGCGCGTGCG <3' and

Probe: 5' > TGATCGAGGAGGAGGTGGCGA <3'

For DNA, a 30 µl reaction mix (50 mM KCl, 10 mM Tris-HCl pH 8.5 at room temperature, 1.5 mM MgCl₂, 0.01% gelatin, 0.5% Tween 20, 0.1% Triton X-100, 1 mM of each primer, 200 mM of each dNTP and 1.25 U of Promega *Taq* polymerase) was used. For cDNA, the same reaction mix with 1.0 mM MgCl₂ was used. For both the DNA template and the cDNA template the cycling parameters were the same. All PCRs used a modified hot start (Erlich *et al.*, 1991) then were heated to 94 °C for 5 minutes of

initial denaturation followed by 50 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 90 seconds.

2.11.6 Optimization of PCR

Initially a standard recommended reaction mix (Gelfand, 1989; Saiki, 1989; Taylor, 1991) was used to amplify 10^3 copies of linearized target plasmid (pGEM-2 vector containing a full-length cDNA of IE1). Then components of the PCR were varied to optimize the amplification. For each individual PCR, more representative template was used for further optimization. These included both genomic DNA from HCMV infected cells and cDNA in RT mix after reverse transcription of RNA from HCMV infected cells. Finally, cycling parameters and the type of tube used for amplification were tested and optimized.

Specific details of optimization are given in each pertinent results sections where PCRs are described.

2.12 ANALYSIS OF PCR-AMPLIFIED DNA or cDNA

2.12.1 Agarose Gel Electrophoresis

Horizontal electrophoresis was performed in 0.6-2% (w/v) agarose gels at 5.0 V/cm. Agarose (ultraPURE™ Agarose, Electrophoresis Grade, BRL) was melted in 1x TBE (0.09M Tris-borate, 0.002M EDTA, pH 8.0) and 0.5 mg/ml of ethidium bromide was added. Samples containing 1x loading buffer (0.025% w/v bromophenol blue, 3.0% v/v glycerol) were loaded and electrophoresis was carried out in 1x TBE buffer. A commercially available 1 kb marker ladder (Gibco, BRL) was used as a molecular weight marker. The electrophoresed samples were examined on a transilluminator (312 nm) and photographed.

2.12.2 Slot Blotting

Four μl of PCR sample were denatured with 1 μl of 5x denaturing buffer (4 M NaCl, 0.4 M NaOH, 0.04 M EDTA, pH 8.0) and heated to 100 °C for 5 minutes then placed on ice immediately before application to a Duralon-UV (Stratagene) nylon membrane. Samples were slot-blotted by suction and each well rinsed twice with 5 μl of 1x denaturing buffer. The membrane was UV fixed (312 nm), DNA-side down, for 5 minutes prior to prehybridization.

2.12.3 Southern Blotting

Agarose gels were denatured (1.5 M NaCl, 0.5 M NaOH) for 45 minutes and neutralized (1.5 M Tris-HCl pH 8.8, 1.5 M NaCl) for 45 minutes prior to incubating in 20 x SSC for 10 minutes. Gels were placed on top of a 3M Whatman wick in a 20x SSC reservoir. A Hybond nylon membrane (Hybond™ N, Amersham), cut to the size of the gel and wet in 2-3x SSC, was placed on top of the gel and bubbles were gently removed by rolling a pipette across the membrane. One wet (2x SSC) and 2 dry 3M Whatman filters, in that order, were placed on top of the nylon membrane. Several layers of paper towels and a weight were placed on top of the 3M Whatman filters and the gel was left overnight to transfer. The next day, the nylon membrane was UV fixed as above (section 2.12.2).

2.12.4 Radio-labeling of Probes

For probing slot blots (section 3.5.1 and 3.5.2), a *Bam*HI/*Bgl*III fragment of the HCMV IE1 cDNA plasmid pJDO83 (Akrigg *et al.*, 1985) that spanned the entire HCMV PCR exon 4 amplification product, was used. The probe was labeled using a random hexanucleotide priming kit (Prime-a-Gene™, Promega).

For probing Southern blots, individual oligonucleotides specific for each PCR amplification product were end-labeled with [³²P] as follows: 300-400 ng of oligonucleotide probe was incubated with 5 µl of 10x blunt-end kinase buffer (0.1 M MgCl₂, 0.5 M Tris-HCl pH 9.5, 50 mM DTT and 50% v/v glycerol), 4 µl of solution X (10 mM spermidine, 0.2 M Tris-HCl pH 9.5 and 1 mM EDTA), 3 µl of gamma [³²P]dATP (3000 Ci/mmol) and 1 µl of T4 polynucleotide kinase (20U/ml) in a total volume of 50 µl. The reaction was incubated for 1 hour at 37 °C, then brought to 100 µl volume with TE. To remove unincorporated dNTPs, 1-2 ml of 50% v/v DE-52 in 0.2 M NaCl/TE was placed in a polyallomer wool plugged 2 ml syringe so that 0.5 to 1 ml of packed DE-52 remained after the NaCl/TE had drained. After settling, the DE-52 was rinsed with 4 ml of TE and the 100 µl of probe was added to the column. Four ml of 0.2 M NaCl/TE wash was used to remove the unincorporated label and the probe was eluted in two, 1 ml aliquots with 0.5 M NaCl/TE. The probe was stored at -70 °C until use.

2.12.5 Prehybridization, Hybridization, Washing & Autoradiography

For slot blotting and for Southern blotting, prehybridization was carried out from 1-16 hours at 42 °C in prehybridization solution (1x Denhardt's solution (2% w/v BSA/Pentax Fraction V, 2% w/v Ficoll, 2% w/v polyvinylpyrrolidone/PVP 40,000 MW, made up in a solution of 3 M NaCl, and 0.3 M trisodium citrate), 5x SSC (0.75 M NaCl, 75 mM trisodium citrate), 2 mM Na₂HPO₄, 2 mM NaH₂PO₄, 50% (v/v) formamide, and 50 mg/ml of herring sperm DNA).

Slot blots were probed at 42 °C overnight with a [³²P]dCTP-radiolabeled 480 bp DNA probe (see above) in 10% (v/v) dextran sulfate/prehybridization solution. Membranes were washed twice in 2x SSC and 0.1% SDS for 15 minutes at room temperature, twice in 1x SSC

and 0.1% SDS for 15 minutes at room temperature, and finally once in 0.1% SSC for 1 hour at 65 °C.

Southern blots were probed with [³²P]-end-labeled specific oligonucleotide probes at 42 °C for 4 hours in 5x SSC, 0.5% (v/v) SDS and 5x Denhardt's solution. Membranes were washed twice in 6x SSC for 10 minutes at room temperature and once in 6x SSC for 5-10 minutes at 55 °C.

All membranes were routinely autoradiographed at -70 °C for up to 72 hours using 2 intensification screens.

2.13 THP-1 CELL LINE

2.13.1 THP-1 Differentiation

THP-1 cells were either treated with preservative-free hydrocortisone sodium succinate (Organon, U.K.) alone, phorbol 12-myristate 13-acetate (Sigma) alone, or in combination. In addition, 1,25 dihydroxy Vitamin D3 alone or in combination with phorbol 12-myristate 13-acetate, as well as granulocyte macrophage-colony stimulating factor in combination with hydrocortisone were used to differentiate THP-1 cells. The use of these agents is detailed in section 5.2.

2.13.2 Infection

Differentiated or undifferentiated THP-1 cells were infected with HCMV strain AD169 (MOI of 10) or mock infected as detailed in section 5.2.5.

2.13.3 Indirect Immunofluorescence

Immunofluorescent analysis using an HCMV anti-IE1 murine Mab is described in detail in section 5.2.10.

2.14 MONOCYTE-DERIVED MACROPHAGE (MDM) PRODUCTION FROM ADHERENT MONOCYTES OF HEALTHY SUBJECTS

2.14.1 MDM Differentiation

Primary monocytes were differentiated to MDM with either phorbol 12-myristate 13-acetate (Sigma) and hydrocortisone sodium succinate (Organon, U.K.), granulocyte macrophage-colony stimulating factor and hydrocortisone sodium succinate or 1,25 dihydroxy Vitamin D3 and phorbol 12-myristate 13-acetate. The use of these agents is detailed in section 5.2.

2.14.2 Infection and Indirect Immunofluorescence

Primary monocytes or MDM were infected with HCMV strain M (MOI of 10) or mock infected as described in detail in section 5.2.9. Immunofluorescent analysis using an HCMV anti-IE1 murine Mab is described in detail in section 5.2.10.

2.14.3 Cocultivation with Fibroblasts

Cocultivation of MDM (differentiated as described above and detailed in section 5.2) with permissive fibroblasts is detailed in section 5.2.11.

CHAPTER 3

SITES OF HCMV PERSISTENCE

3.1 INTRODUCTION

Many aspects of the biology and pathogenesis of HCMV are poorly understood. One area of uncertainty involves the site of virus persistence in the healthy seropositive individual. HCMV infection clearly can be transmitted by blood products from healthy donors to susceptible recipients (Adler, 1983; Tolpin *et al.*, 1985). Moreover, the incidence of transfusion-associated HCMV infection can be markedly reduced by using WBC depleted blood products (Yeager *et al.*, 1981; Gilbert *et al.*, 1989; De Grann-Hentzen *et al.*, 1989, DeWitte *et al.*, 1990). These reports, therefore, suggested WBC as at least one possible site of HCMV persistence.

However, other than one report (Diosi *et al.*, 1969), HCMV has never been isolated from the buffy coat of healthy donors, even though a cumulative total of over 1500 buffy coats from blood donors was analyzed in 4 different studies (Perham *et al.*, 1971; Mirkovic *et al.*, 1971; Kane *et al.*, 1975; Bayer & Tegtmeier, 1976; Jordan, 1983). In addition, it was not possible to detect HCMV sequences by dot blot analysis even using 10 µg of human DNA from healthy HCMV seropositive subjects (Saltzman *et al.*, 1988). Thus, although circumstantial evidence indicated that virus must be present in WBC of healthy carriers, it would seem to be present in extremely low copy number.

Using more sensitive techniques, one group (Schrier *et al.*, 1985), using in situ hybridization with an AD169 *EcoRI* J fragment probe, reported evidence of HCMV transcripts in PBMC (primarily T cells) of healthy seropositive and some seronegative subjects. In contrast, Gnann *et al.* (1988), using a similar in situ hybridization technique with the same

IE probe as well as a late probe (*Bam*HI R fragment of strain AD169), found no detectable HCMV signal in any healthy pre-transplant donor kidneys from seropositive subjects despite the fact that donor lymphocytes would have been present in the organs and even though transplanted kidneys from seropositive donors have the ability to transmit virus to seronegative and seropositive recipients (Chou, 1986; Grundy *et al.*, 1988; Grundy *et al.*, 1987). Consequently, it would appear that in situ hybridization is also not sensitive enough to detect such rare DNA or RNA copy numbers.

Perhaps the most sensitive method of examination which has been applied to the detection of HCMV sites of persistence is the polymerase chain reaction. There have been 3 previous reports of detection of HCMV in PBMC of healthy carriers using the PCR technique (Stanier *et al.*, 1989; Bevan *et al.*, 1991; Stanier *et al.*, 1992). However, there was no attempt in any of these publications to analyze specific cell populations harboring HCMV.

Finally, although there have been a number of reports using the PCR technique to detect HCMV in clinically ill patients, these reports have failed to detect HCMV in healthy carriers (Shibata *et al.*, 1988; Jiwa *et al.*, 1989, Rowley *et al.*, 1991, Gerna *et al.*, 1991) with one exception (Cassol *et al.*, 1989). Cassol *et al.* (1989), found 1 of 10 healthy seropositive control subjects was PCR positive. These analyses indicated that although PCR in general was noted to be a sensitive technique, for the detection of low copy number template it would require a fully optimized PCR methodology and most likely nested amplification with blotting and probing of final products to detect low copy number sequences in healthy subjects.

Thus, whilst HCMV must be present in peripheral blood leukocytes, the exact cell type harboring HCMV was unclear. I therefore decided to use highly purified populations of cells combined with a sensitive

amplification technique, PCR, (Saiki *et al.*, 1985; Saiki *et al.*, 1986; Mullis & Faloona, 1987; Saiki *et al.*, 1988; Erlich *et al.*, 1988) to attempt to define the specific site of persistence of HCMV in the peripheral blood of healthy asymptomatic carriers. I began by fractionating cells from the peripheral blood of healthy asymptomatic carriers into CD3 bearing PBMC (T cells), non-CD3 bearing PBMC (non-CD3 T cells, B cells, and monocytes), adherent PBMC (highly enriched monocyte population) and CD14+ PBMC (monocytes). Following this, CD4+ and CD8+ T cells were also examined.

Ultimately, because detection of HCMV had been reported in the polymorphonuclear leukocyte fraction (PMNL) of peripheral blood from patients infected with HIV, other immunocompromised patients, and some immunocompetent patients acutely ill with HCMV (Fiala *et al.*, 1975; Rinaldo *et al.*, 1977; Saltzman *et al.*, 1988; Dankner *et al.*, 1990; Gerna *et al.*, 1991; Revello *et al.*, 1992), I also examined PMNLs of healthy carriers to determine whether the granulocyte might also represent a normal site of persistence which would be consistent with these clinical observations.

3.2 MATERIALS AND METHODS

General materials and methods are detailed in Chapter 2.

3.2.1 Preparation of DNA and Controls for Seronegative Subjects

DNA from specific cell types of healthy seropositive subjects was isolated as described in Materials and Methods section 2.7. However, specifically for the analysis of seronegative subjects, heparinized blood and similar volumes of control solutions (PBS containing the same batch and concentration of heparin) were processed side by side on Lymphoprep™ gradients to yield PBMC and controls. This additional control was used as a further precaution to detect any possible false positive results due to amplification of contaminating DNA. PBMC and controls were divided

into 3 aliquots and frozen at -70°C to be processed into DNA on separate occasions, as described in Chapter 2 using the RNase and Proteinase K treatment followed by phenol extraction.

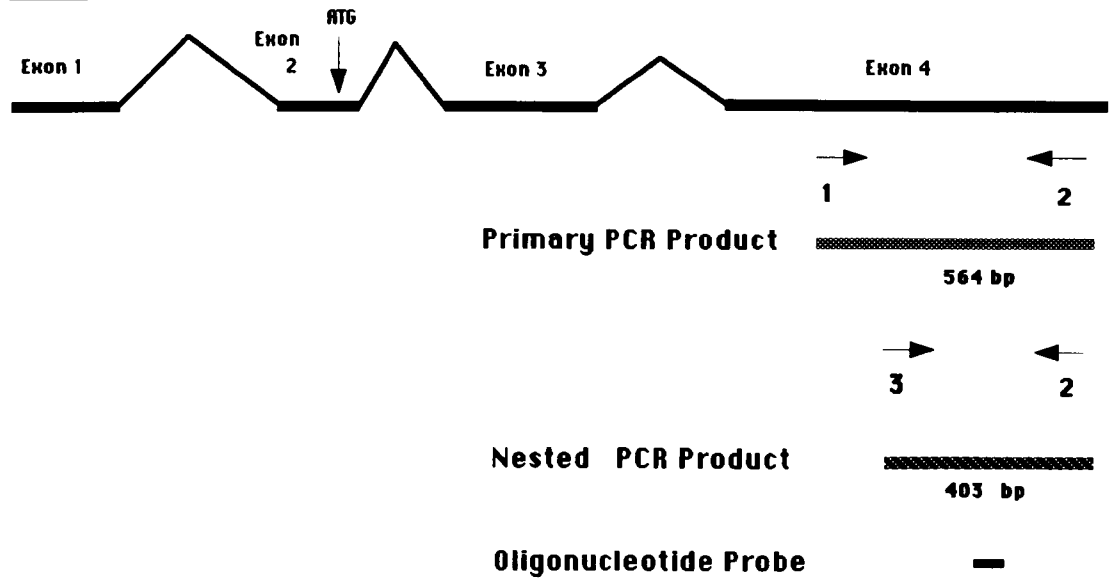
3.2.2 HCMV Immediate Early Gene PCRs

The HCMV Immediate Early Gene PCR was an asymmetric-nested amplification. The term asymmetric-nested is used here to describe the use of a single anti-sense primer paired with a sense primer for the initial 30 cycle amplification and then the same anti-sense primer paired with a more internal sense primer for the secondary or nested 30 cycle amplification. The sequences of the primers and probe and a diagrammatic representation of their locations within IE1 are shown in Figure 3.1. All were located in exon 4 (Akrigg *et al.*, 1985).

Alternatively, a fully nested PCR (Mullis & Faloona, 1987; Bell, 1989; Jackson *et al.*, 1991) was used. The sequences and locations of the primers and the probe used for this PCR are shown in Figure 3.2.

Optimized PCR parameters (detailed in section 3.3.1) for both PCRs were as follows: a 50 µl reaction mix contained 50 mM KCl, 10 mM Tris-HCl pH 8.5 at room temperature, 2 mM MgCl₂, 0.01% gelatin, 0.5% Tween 20, 1 mM of each primer, 200 mM of each dNTP and 1.25 U of *Taq* polymerase (Perkin Elmer Cetus, Native *Taq*). The primary PCR routinely amplified 1 µg of DNA. For the nested amplifications, 2 µl of the first reaction mix was used as a template. For both PCRs the cycling parameters were the same. Amplifications were performed on a Perkin Elmer Cetus Thermocycler and were 94 °C for 5 minutes of initial denaturation followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 90 seconds.

3.1A



3.1B

Sense primer:

5' > CTGCAGAACTTGCCTCAGTGCTCCCCTGAT < 3'

Anti-sense primer:

5' > GTTCTCAGCCACAATTACTGAGGACAGAGG < 3'

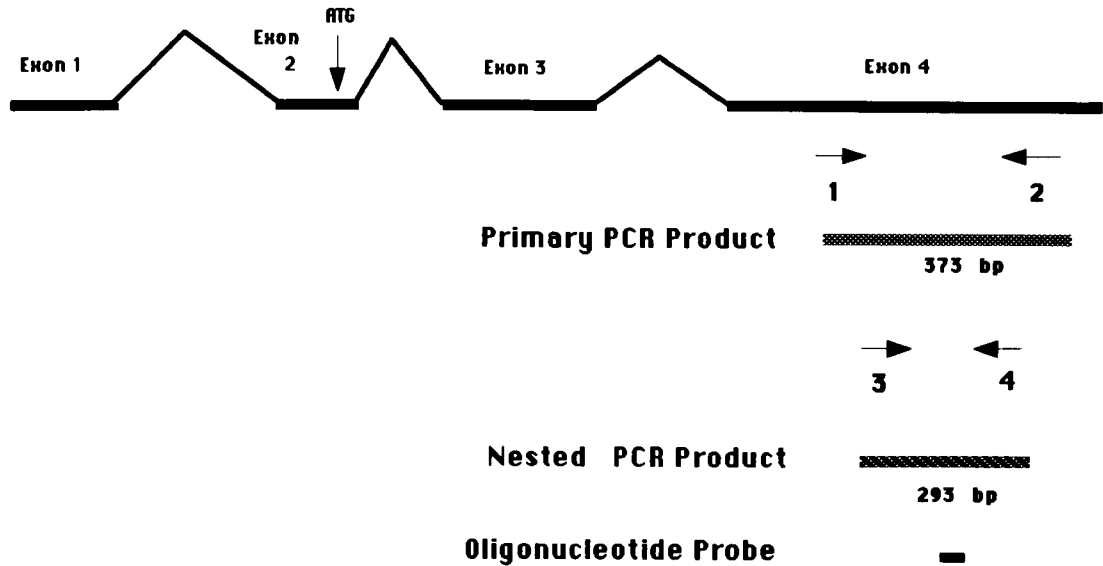
Nested sense primer:

5' > GGTCACTAGTGACGCTTGTATGATGACCATGTACGG < 3'

Probe: 5' > GCCGCATTGAGGAGATCTGCATGAAGGTCT < 3'

Figure 3.1. HCMV IE1 Asymmetric-Nested PCR. Panel A shows the organization of the HCMV IE1 region at the top (thick lines representing exons and thin lines representing introns) and at the bottom, the HCMV IE1 asymmetric-nested PCR. This PCR initially amplified a 564 bp product (30 cycles) using a sense (arrow 1) and anti-sense (arrow 2) primer from exon 4. This was followed by a second amplification (30 cycles) using a more internal sense primer (arrow 3) but the same anti-sense primer as in the first 30 cycles. The final product was 403 bp. (Panel B) Primer sequences are listed. For probing, an oligonucleotide internal to the secondary nested primers was used.

3.2A



3.2B

Sense primer:

5' > GGTCACTAGTGACGCTTGTATGATGACCATGTACGG < 3'

Anti-sense primer: 5' > GATAGTCGCGGGTACAGGGGACTCT < 3'

Nested sense primer: 5' > AAGTGAGTTCTGTCGGGTGCT < 3'

Nested anti-sense primer: 5' > GTGACACCAGAGAATCAGAGGA < 3'

Probe: 5' > GCCGCATTGAGGAGATCTGCATGAAGGTCT < 3'

Figure 3.2. HCMV IE1 Nested PCR. (Panel A) The organization of the HCMV IE1 region is shown at the top and the design of the HCMV IE1 fully nested PCR is shown below. This nested PCR consisted of two 30 cycle sets of amplification and initially yielded a 373 bp product with a secondary 293 bp product. All primers used for the HCMV amplification were located in exon 4 and were: primary sense primer (arrow 1), primary anti-sense primer (arrow 2), nested sense primer (arrow 3) and nested anti-sense primer (arrow 4). A probe internal to the secondary primer pair was prepared for blotting.

(Panel B) Primer and probe sequences are shown.

3.2.3 HCMV Early Gene PCR

For experiments in sections 3.4.4 and 3.4.5 an early gene PCR was used. This amplified a 315 bp portion of the major early gene (Greenaway & Wilkinson, 1987). The sense primer, anti-sense primer and internal oligonucleotide probe, respectively, were:

5' > CGTTATCCGTTCTCGTAGG < 3',

5' > GTTTCGTTGTTGTCGGTAGT < 3' and

5' > CCTACCACGAATCGCAGATGA < 3'.

PCR parameters optimized (see section 3.3.1) for the early PCR were the same as for the IE1 PCRs detailed above except, a 30 µl reaction mix was used with 2.5 mM MgCl₂, 0.1% Triton X-100 and 0.75 U of *Taq* polymerase (Promega). In addition, a modified hot start was used (detailed in section 3.3.1). The cycle parameters were the same as above except 50 cycles were used.

3.2.4 Histidyl-tRNA Synthetase PCR

The histidyl-tRNA synthetase PCR amplifies a multi-copy housekeeping gene and was used as a control PCR for the general ability to amplify DNA. A detailed description is given in section 2.11.5.

3.3 RESULTS: PCR DETECTION

3.3.1 Results of PCR Optimization

General optimization parameters are discussed here, while specific results are detailed with the description of each individual PCR. Both low copy number HCMV infected cell DNA (which would be most like the *in vivo* situation) as well as plasmid targets and cDNA from RT preparations were tested.

First, KCl concentration (0, 30, 50 & 60 mM) and 10 mM Tris-HCl pH (titration of pH 8.3, 8.4, 8.5, 8.7 at 25 °C) were optimized. Greater than 75

mM KCl is reported to significantly decrease *Taq* activity, while enzyme activity varies with lesser concentrations depending on the specific PCR (Innis *et al.*, 1988; Gelfand, 1989; Yang *et al.*, 1989). Optimal activity of *Taq* has a fairly broad pH range 8.2-9.0 at 25 °C in 10 mM Tris (Yang *et al.*, 1989; Taylor, 1991). My analysis (Figure 3.3, top lanes 1-8) showed that 50 mM KCl, 10 mM Tris-HCl pH 8.5 at room temperature was optimum and these values have been used to successfully amplify products in all PCRs used in this analysis.

The presence or absence of gelatin was also tested. Gelatin has been recommended, although one report has suggested that it consistently decreased PCR yields when using a plasmid target (Yang *et al.*, 1989). For the IE PCRs, 0.01% gelatin increased detection of genomic template (Figure 3.3, top lanes 9 &10) but had no effect on detection of a plasmid template.

The use of combinations of detergents (including NP-40, Tween 20, and Triton X-100) was also tested. It has been reported that detergents reverse inhibitory effects of SDS, which can inhibit up to 10% of baseline dNTP incorporation when present at a concentration of 0.01% (Gelfand, 1989). In addition, other work suggests that some detergent is essential to obtain maximum processivity of *Taq* and to reduce the background caused by false terminations from the enzyme (Innis *et al.*, 1988; Taylor, 1991). In all PCRs, it was found that NP-40 had no effect, whereas 0.5% Tween-20 in combination with 0.1% Triton X-100 was optimum (Figure 3.3, top lanes 11-14). The 2 intra-exon 4 HCMV IE1 PCRs used in this chapter employed only Tween 20. It was later in my study that I found Triton X-100 to be optimal in combination with Tween 20.

Primer concentrations and dNTP concentrations were used as recommended and were not optimized (Saiki, 1989; Taylor, 1991). The concentration of dNTPs used was held constant for all MgCl₂ titrations.

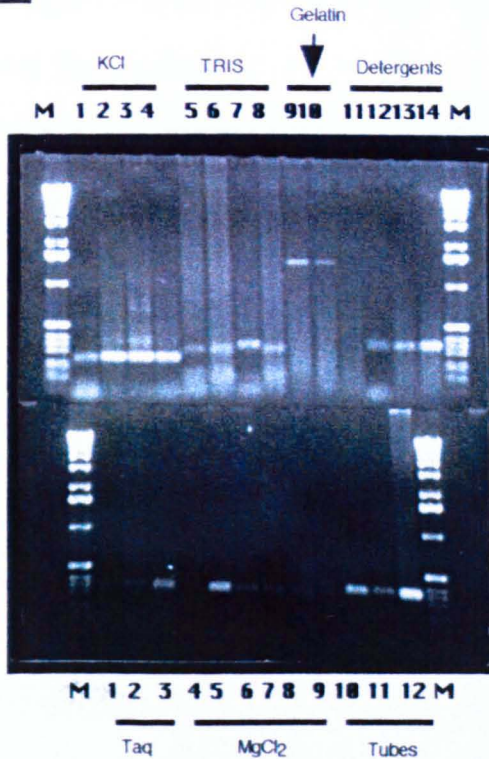
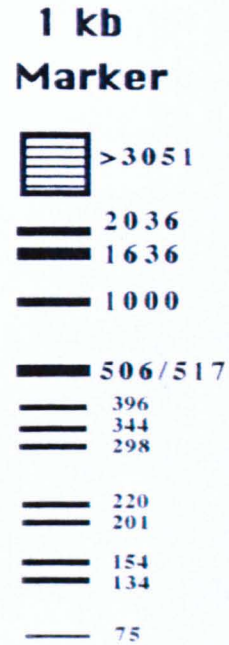
3.3 A3.3 B

Figure 3.3. Optimization of PCRs. (A) The ethidium bromide gel shows the results of optimization of 7 variables in representative PCRs. The top portion of the gel shows optimization of KCl (lanes 1-4 using a nested IE2 RT-PCR detailed in 4.2.3; 266 bp cDNA product), of pH of Tris-HCl (lanes 5-8 using a primary IE1 RT-PCR detailed in 4.2.2; 353 bp cDNA product), of gelatin (lanes 9-10 using a primary IE1 PCR detailed in 4.2.2; 1464 bp DNA product), and of detergents (lanes 11-14 using a primary IE1 RT-PCR detailed in 4.2.2; 353 bp cDNA product). Specifically, lanes 1-4 = 0, 30, 50 and 60 mM KCl; lanes 5-8 = Tris-HCl pH 8.3, 8.4, 8.5 and 8.7; lanes 9-10 = 1% and no gelatin and lanes 11-14 = no detergents, 0.5% Tween 20, 0.1% Triton X-100 and 0.5% Tween 20 + 0.1% Triton X-100, respectively. The bottom of the gel shows the optimization of *Taq* products (lanes 1-3 using a primary IE1 RT-PCR detailed in 4.2.2; 353 bp cDNA product), of MgCl₂ (lanes 4-9 using a primary IE1 RT-PCR detailed in 4.2.2; 353 bp cDNA product) and of PCR tubes (lanes 10-12 using a histidyl-tRNA synthetase PCR; 370 bp DNA product detailed in 2.11.5). Specifically, lanes are: lanes 1-3 = Beckman *Taq*, Perkin Elmer Cetus native *Taq* and Promega *Taq*; lanes 4-9 = 0.5, 1.0, 1.5, 2.0, 2.5 & 3.0 mM MgCl₂ and lanes 10-12 = Sarstedt, Treft and Perkin Elmer Cetus GeneAmp™ tubes. (B) All lanes marked M are the 1 kb DNA marker (Gibco) with specific size bands as detailed.

This was important as dNTPs are known to quantitatively bind free Mg^{2+} (Saiki, 1989; Taylor, 1991).

Taq concentration (0.5 U to 4 U/100 μ l reaction mix) as well as different *Taq* enzymes by various manufacturers were tested (Figure 3.3, bottom lanes 1-3). For low copy number target, 2.5 Units per 100 μ l of reaction mix of Promega native *Taq* was most successful. The 2 intra-exon 4 IE1 HCMV PCRs used in this chapter employed Perkin Elmer Cetus native *Taq* because it was only later that I determined Promega *Taq* to be optimal for low copy target.

Optimum $MgCl_2$ was evaluated by amplifying specific, low copy number template in varying dilutions ranging from 0.5-3.0 mM at 0.5 mM increments (Figure 3.3, bottom lanes 4-9). In general, excess Mg^{2+} is reported to result in increased mispriming events, whereas insufficient Mg^{2+} will reduce the yield of products (Saiki, 1989). It was particularly important to always use specific template in low copy number (so that a clear optimum was observed), while holding concentration of dNTPs constant (see above). Optimum $MgCl_2$ concentration varied from PCR to PCR with a range of 1-2.5 mM $MgCl_2$ used for the PCRs in this analysis.

Cycle parameter optimization was tested. The temperature for denaturing, 94 °C, was used for genomic templates and cDNA. *Taq* half-life at 94 °C is about 40 minutes (Gelfand, 1989). For a 50-cycle amplification with 30 seconds of denaturation per cycle, *Taq* undergoes less than 1 half life. The annealing temperature was determined by the base content of the primers (for 20 mer oligonucleotides): $T_{annealing} = T_{melting} (4x G+C) + (2x A+T) - 5$ °C. Time for annealing was found to be 30 seconds and, although longer times were just as efficient, 30 seconds was found to reproducibly amplify product. Shorter time intervals were not tested because it is reported to take a minimum of 20 seconds to equilibrate a 100 μ l reaction mix in the Perkin Elmer Cetus heating block

using 0.5 ml GeneAmp™ tubes. The extension temperature was determined by the optimum growth temperature for the Thermus aquaticus bacterium, 70-75 °C (Saiki, 1989). The optimum time for the extension phase was 90 seconds (times tested: 0.5 to 3 minutes in 30 second intervals). Any time less than 90 seconds was not efficient for all PCRs, even though *Taq* synthesizes DNA at a rate of 1 kb per minute (Saiki, 1989). Any time greater than 90 seconds showed no improvement.

Although the temperature optimum of *Taq* polymerase is 70-75 °C it is able to function at lower temperatures and is as efficient as Klenow at 37 °C. For this reason, nonspecific DNA synthesis from mis-priming (primers annealing to non-target sequences particularly at low temperatures) and primer-dimer annealing (primers annealing from 3' complementarity without any other DNA target necessary) can occur. These reactions compete for target-specific amplification and can markedly reduce PCR specificity and sensitivity. To eliminate this potential problem, all PCRs used a modified hot-start such that the complete PCR mix was placed on ice immediately after adding template, then transferred directly to a hot (94 °C) PCR machine block to begin denaturation (Haff & Atwood, 1989; Erlich, *et al.*, 1991).

Using temperature optimums, determined above, the type of tube used in the Perkin Elmer Cetus machine was optimized. Three types of tubes were tested with DNA template and RT cDNA template (Figure 3.3, bottom lanes 10-12). It was found that the GeneAmp™ reaction tubes (Perkin Elmer Cetus) were optimal. The latter tube is designed specifically to fit the Perkin Elmer Cetus Thermocycler. Correct fitting of tubes in the sample block aids the efficiency of heat transfer to and from the sample and most likely influenced effectiveness of amplification. In addition, no volume larger than 50 µl was used so that temperature equilibration was

rapid, particularly to maximize the hot starting procedure described above.

3.3.2 PCR Sensitivity and Specificity

Sensitivity of the asymmetric IE1 PCR was 10 copies and the fully nested IE1 PCR was 1 copy of linearized pGEM-2 IE1 plasmid (Figure 3.4). The early gene PCR was able to detect 10 femtograms of HCMV AD169 infected cell DNA. As determined in Figure 4.5, comparing the amplification level of this same HCMV DNA stock to a known copy number plasmid control showed that 10 femtograms of DNA was approximately equal to 10 copies. Thus, the HCMV early gene PCR sensitivity had a level of detection equal to 10 copies.

All primers amplified an area of the HCMV genome which has no complementarity to that of human DNA or other herpesviruses (Shaw *et al.*, 1985, Chee *et al.*, 1990).

3.3.3 PCR Detection of Viral Variants

These primers were used successfully to amplify HCMV DNA from six clinical HCMV isolates, from the AD169 strain, and from 20/20 healthy seropositive subjects.

3.4 RESULTS: SITES OF PERSISTENCE

3.4.1 Serology

All subjects were unequivocally seropositive or seronegative. Seropositive values for the competitive ELISA ranged from 4%-20% (positive values <50%), and 82-104% (negative values >66%). For all seronegative subjects a confirmatory latex agglutination was done to rule out early primary infection. The latex agglutination assay detects IgM as well as IgG antibody. For all seronegative subjects, found to be PCR +, a

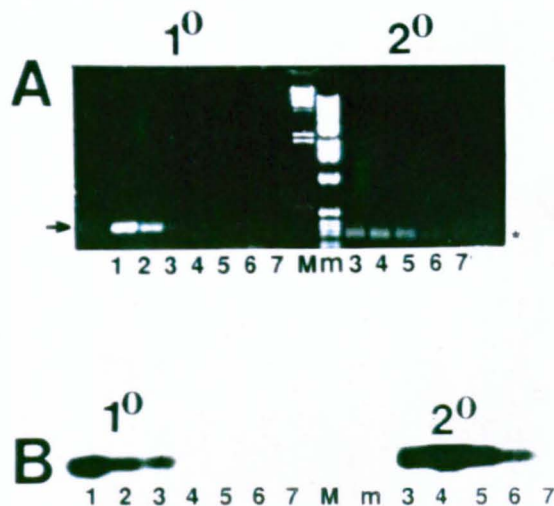


Figure 3.4. Sensitivity of HCMV IE1 Nested PCR. Panel A is an ethidium bromide-stained agarose gel showing the amplification products from the primary 30 cycle HCMV PCR of 10^6 , 10^4 , 10^3 , 10^2 , 10^1 , and 1 copy of linearized pGEM-2 IE1 plasmid and a non-DNA containing negative control, respectively (labeled 1^0 : lanes 1 to 7). The secondary HCMV PCR (labeled 2^0 : lanes 3 to 7) shows the nested amplification of 10^3 , 10^2 , 10^1 , and 1 copy of linearized pGEM-2 IE1 plasmid and a non-DNA-containing negative control from the primary PCR, respectively. The marker in lane M is a *Hind*III digest of lambda phage (Northumbria Biological); the marker in lane m is a 1 kb ladder (Gibco BRL). The arrow indicates the 373 bp primary amplification products and the asterisk, the 293 bp secondary amplification products. Panel B is the Southern blot analysis of the gel in (A) probed with an HCMV-specific oligonucleotide.

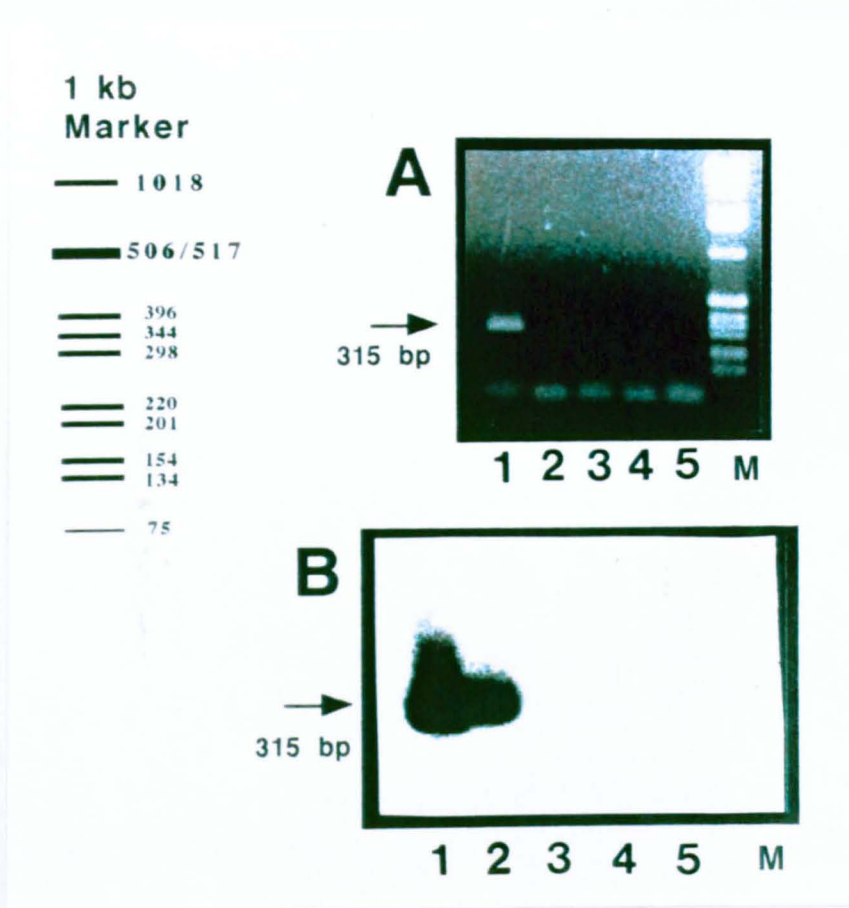


Figure 3.5. Early Gene PCR Sensitivity. The ethidium bromide gel in (A) and the Southern blot in (B) show the results of an early gene PCR amplification of dilutions of a standard stock DNA control from HCMV AD169 infected cells. Lanes 1- 5 show the amplification of the following dilutions of the standard HCMV infected cell DNA control: 1×10^{-6} (100 femtograms), 1×10^{-7} (10 femtograms), 5×10^{-8} (5 femtograms), 1×10^{-8} (1 femtogram) and no DNA H₂O control, respectively. The limit of detection in this PCR is 10 femtograms of DNA as shown by the 315 bp product seen in (A) lane 2 and the as shown by the probe specific Southern blot in (B) lane 2. The 1 kb DNA marker (lanes M) is detailed at the top left.

second serum was obtained 2-6 months after the initial one. All samples remained seronegative by latex agglutination and ELISA.

3.4.2 HCMV DNA is Present Primarily in CD3-, CD14+, and Adherent PBMC of Asymptomatic Seropositive Individuals

PBMC from 6 seropositive and 2 seronegative individuals were separated by FACS using a CD3 marker for T cells. Purity analyses of sorted populations of PBMC were performed on cells derived from these eight sorts. Purity ranged from 93.8 to 99.77% cells in the positively sorted populations and from 94 to 99.6% cells in the negatively sorted populations.

For all 8 individuals, DNA was extracted from the sorted PBMC by RNase/Proteinase K method and equal amounts (1 µg) of the CD3+ and CD3- populations were amplified by asymmetric-nested PCR for HCMV IE1. For 5 of 6 seropositive subjects (Figure 3.6 panel A, S1, S2, S3, S5 & S6), autoradiography of slot blot hybridization analyses reproducibly showed CD3- signal greater than CD3+ signal. The remaining seropositive subject (S4) showed equal signal in the CD3+ and CD3- cells. All DNAs, however, showed equal amplification in the histidyl-tRNA synthetase PCR (Figure 3.6, panel B). For the 2 seronegative subjects, one (Figure 3.6, S8) showed no HCMV signal in either the CD3- or CD3+ cell DNAs while the other (Figure 3.6, S7) showed the same pattern as that of 5 of 6 seropositive individuals, that is, the predominant HCMV positive signal was seen in the CD3- cells.

To determine if the positive HCMV IE1 signal in the CD3- cells was due to monocytes, DNA from adherent cells from 9 seropositive individuals was examined by PCR and Southern blotting. Purity analysis was derived from 6 subjects. Anti-CD14 and anti-CD3-specific antibodies were used to examine adherent cells by indirect immunofluorescence. In all cases the adherent cells showed specific fluorescence for the CD14 marker, with less

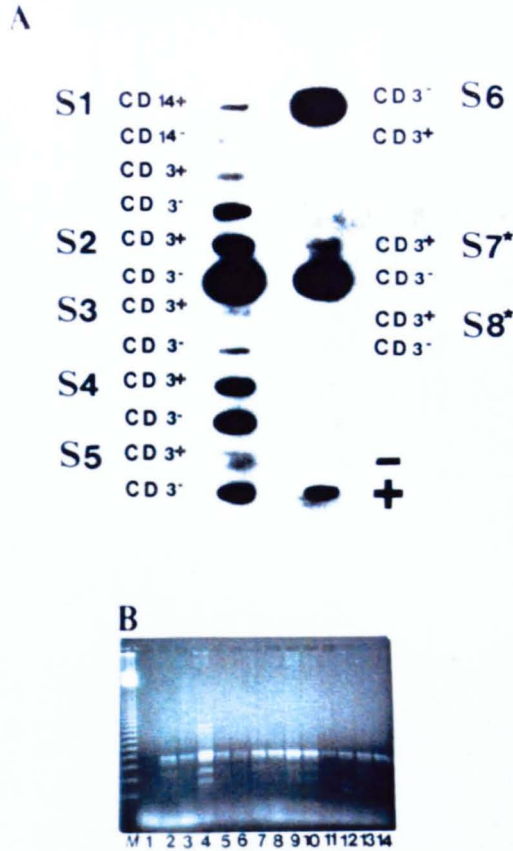


Figure 3.6. Detection of HCMV Sequences in Specific FACS Populations.

Panel A shows an autoradiograph of PCR products of 6 seropositive subjects (S1 to S6) and two seronegative subjects (S7* and S8*) after slot blot analysis. A positive control of 100 pg pES (Boom *et al.*, 1986) blotted directly without PCR amplification (+) and a non-template negative control (-) is also shown. Cell populations are identified as CD14+ or CD14-, and CD3+ or CD3-. Panel B shows DNA from subjects in (A) analyzed by histidyl-tRNA synthetase PCR. Amplified products were separated on agarose gels and stained with ethidium bromide. Although some mis-primed smaller bands can be seen, the prominent 370 bp band was clearly seen as the upper-most product in lanes 2-14. Subjects are: S1 CD14+ and CD14- (lanes 2 and 3), S2 CD3+ and CD3- (lanes 4 and 5), S6 CD3+ and CD3- (lanes 6 and 7), S3 CD3+ and CD3- (lanes 8 and 9), S7 CD3+ and CD3- (lanes 10 and 11), and S8 CD3+ and CD3- (lanes 12 and 13). Lane 1 is a reaction mix negative control and lane 14 is a DNA positive control. Lane m, is a 100 bp ladder (Pharmacia).

than 5% of cells positive for the CD3 marker, indicating a highly pure population of mature monocytes. After PCR amplification an HCMV-specific band was detected in all 9 subjects. Two subjects are shown in Figure 3.7 and a further 7 are shown in Figure 3.10. Similar to the findings in the CD3+ sorts, non-adherent cells showed significantly less or no HCMV-specific PCR signal as compared to adherent cells.

As cells which adhere to plastic may not consist entirely of monocytes (Hunt, 1987), PBMC from one individual were sorted by FACS into CD14+ and CD14- cells (Figure 3.6 panel A, S1). CD14 is a marker for mature monocytes. DNA was then amplified by asymmetric-nested PCR and analyzed by slot blot hybridization and probing. CD14+ cells gave a positive signal whereas CD14- cells were negative, and histidyl-tRNA synthetase PCR (Figure 3.6 panel B, lanes 2-3) amplified both these samples equally.

3.4.3 HCMV DNA is Present in Some Seronegative Subjects

In 6 of 9 seronegative subjects, the data for 6 of which are shown, HCMV IE1 PCR results were repeatedly negative by gel electrophoresis, and Southern blot analysis of at least 2 DNA samples prepared separately (Figure 3.8). However, in three subjects, from at least two DNA samples prepared separately, HCMV PCR results were positive whilst comparable control samples were negative (Figure 3.8). All samples and controls were processed using the RNase/Proteinase K method (2.7.1). For the 3 seronegative but PCR positive individuals, new specimens of PBMC DNA were obtained and reanalyzed. On re-analysis of at least 2 separately prepared DNA samples from these 3 individuals, HCMV PCR remained positive. One of these individuals was analyzed further by cell sorting to yield T cell (CD3+) and non-T cell (CD3-) PBMC populations (Figure 3.6 Panel A, S7). The HCMV PCR results showed that the non-T cell



Figure 3.7. Detection of HCMV Sequences in Adherent PBMC. (A) Ethidium bromide-stained agarose gel showing the amplification products from adherent cells from 2 seropositive subjects (lanes 1 and 2) and PBMC from a seronegative subject (lane 3) after fully nested PCR. DNA was extracted using RNase/Proteinase K method (section 2.7.1). Lane 4 contains 10^4 copies of pGEM2 IE1 and lane 5 represents a non-DNA-containing reaction mix, negative control. The specific 293 bp amplification product is marked by an arrow. Lane m is a 1 kb marker ladder (Gibco, BRL). Smaller bands generated by oligonucleotide primer dimers or mis-priming events are not probe-positive. (B) Southern blot analysis of the gel in (A) probed with an HCMV-specific oligonucleotide probe.

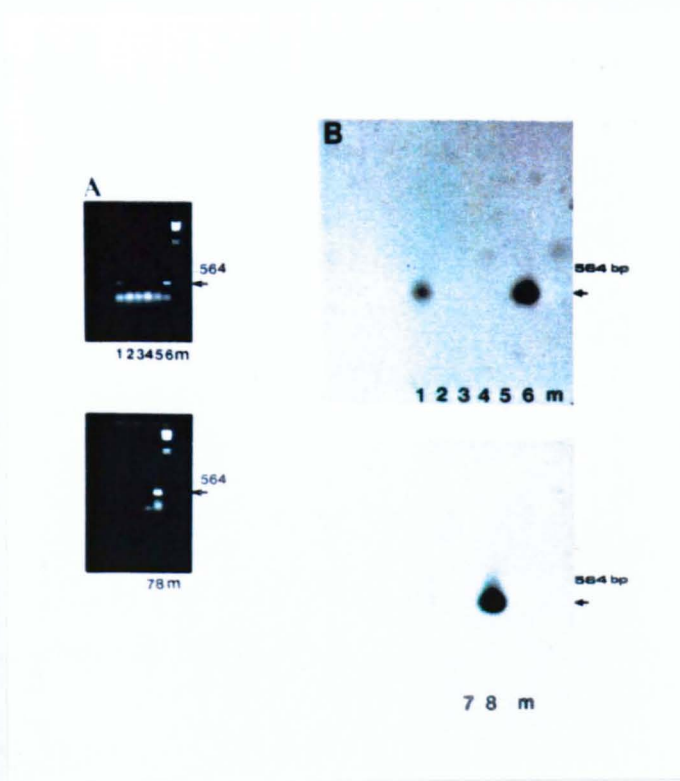


Figure 3.8. HCMV Sequences in PBMC of Healthy Seronegative Carriers.

(A) Panel A shows an ethidium bromide-stained agarose gel after fully nested IE1 PCR of DNA prepared from PBMC of 6 seronegative healthy subjects (lanes 1 to 6), a non-template negative control (lane 7) and a positive control (lane 8; DNA prepared from PBMC of a seropositive healthy subject). Lane m is a *Hind*III digest of lambda phage and shows the 564 bp band. The arrow indicates the specific 293 bp product. Smaller bands are oligonucleotide primers and are not probe-positive. (B) Panel B shows the autoradiograph of the gel in (A) after Southern blotting and labeling with an HCMV-specific oligonucleotide probe. The arrow indicates the probe-positive 293 bp amplification product.

population harbored the HCMV signal, as determined by slot blot hybridization analysis and probing. These results were comparable to the findings in 5 of 6 seropositive subjects, in whom HCMV DNA was present, predominantly in CD3⁻ cells.

3.4.4 HCMV is Present in Some CD8⁺ T Lymphocytes

Whilst HCMV DNA was predominately found in the CD3⁻ cells, 2 of the 7 HCMV carriers analyzed in Figure 3.6 (S1 & S2) showed evidence of weak signal or, in one case, equal signal in the CD3⁺ population as compared to the CD3⁻ population. Consequently, to further investigate whether HCMV could be detected in a subpopulation of CD3⁺ T cells, purified populations of T cells were examined. For one individual (subject S1 in Figure 3.6), bead-sorted CD4⁺ and CD8⁺ cells as well as adherent monocytes were analyzed for the presence of HCMV IE1 DNA (Figure 3.9).

Purity analysis of CD8⁺ and CD4⁺ cells was analyzed after examining cytopun cells. Although in most cases there were still beads attached to cells (despite the use of DetachaBead), positive fluorescence was observed with the same antibody used to positively select the cells. In addition, many cells were fragmented (most likely due to residual beads) but a minimum of 50 intact cells per spot was able to be counted. For the CD8 and CD4 positive selections analyzed with CD8 and CD4 antibody, respectively, there were no intact cells observed without positive membrane fluorescence. For CD4⁺ or CD8⁺ selected cells analyzed with CD8 or CD4 antibody, respectively, there were rare cell membrane fragments and intact cells seen. None of the cells were positive for butyrate esterase.

All DNAs were extracted using a sodium perchlorate method (2.7.2). Equal amounts of DNA were amplified in the HCMV early gene PCR and the control PCR, histidyl-tRNA synthetase. Results of the latter PCR

showed all DNAs to be equally amplified (Figure 3.9 B). Adherent monocytes were positive on both ethidium bromide gel electrophoresis and Southern blot, while the CD8+ cells were HCMV positive on Southern blot only and the CD4+ population showed no evidence of HCMV DNA even after blotting and probing.

3.4.5 HCMV is Not Present in Polymorphonuclear Cells of Asymptomatic Subjects

From each of 10 subjects, PMNL enriched cells and adherent monocytes were analyzed to determine if PMNLs were a site of HCMV persistence. Purity analysis of PMNL enriched cell fractions after butyrate esterase staining showed: differential cell counts yielded a range of 90-99% PMNLs (mean = 95%); all other cells were mononuclear yet none were positive for butyrate esterase, therefore, these latter cells were considered to be lymphocytes not monocytes. Purity analysis of the adherent cells, isolated simultaneously with the PMNL-enriched fractions, showed the population to be largely monocytes with 92-97% butyrate esterase positive.

In 7 of 7 seropositive healthy subjects analyzed in at least two experiments, adherent PBMC DNA was HCMV PCR positive while PMNL DNA was negative (Figure 3.10). All DNAs were extracted using the sodium perchlorate method. Adherent PBMC DNA was HCMV PCR positive for the one seronegative subject from whom DNA was prepared from two separate samples obtained 2 months apart (Figure 3.10, lane 15). In this latter individual the results were confirmed by another HCMV PCR using primers in a region of the glycoprotein B gene for HCMV (Carolyn Tysoe, unpublished observation). Five of the 8 HCMV PCR positive subjects showed a discernible PCR product of the correct size on ethidium bromide-stained agarose gel, while the other 3 were positive by Southern blot only (Figure 3.10 B). The other 2 seronegative subjects were

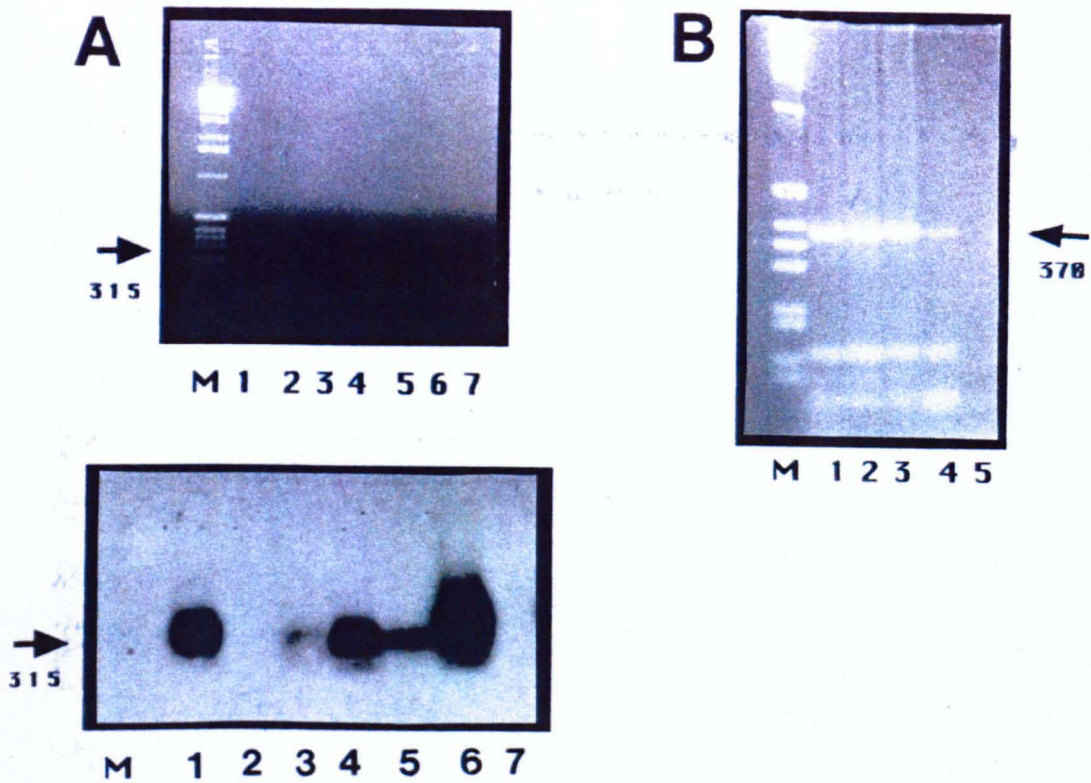


Figure 3.9. HCMV PCR Analysis of Bead-Purified T Lymphocytes.

Analysis of bead-purified T lymphocytes is seen in panel A (HCMV PCR) and panel B (control PCR). (A) shows the ethidium bromide-stained gel where lanes 2, 3 and 4 are the early gene amplifications from the test subject (S1, Figure 3.6). Lane 2 is CD4⁺ T cells, lane 3 is CD8⁺ T cells and lane 4 is adherent monocytes. Lane M is a DNA marker (1kb ladder, Gibco) and the controls are seen in the following lanes: lane 6 is 10³ copies of HCMV DNA, lanes 1 & 5 are 1 μ g of adherent monocyte DNA from 2 other healthy carriers and lane 7 is a non-DNA negative control. The arrow denotes the size of the amplified products for the HCMV early gene PCR (315 bp). The Southern blot of the HCMV early gene PCR after oligonucleotide probing is shown below the agarose gel. Panel B shows the histidyl-tRNA synthetase PCR for the bead-sorted T cells and the adherent monocytes from the test subject shown in Panel A (lanes 2,3,4). Lane M is a DNA marker (1kb ladder), lanes 1,2,3 are 100 ng of DNA from the CD4⁺, CD8⁺, and adherent monocytes of the test subject, respectively. Lanes 4 and 5 are the positive (10 ng of human DNA) and negative controls (no DNA), respectively. The arrow denotes the 370 bp amplification product for the house-keeping gene/control PCR.

consistently negative for HCMV DNA in both cell populations, even after Southern blot and probing (Figure 3.10).

For all DNA samples analyzed, equal amounts of DNA (100 ng) were amplified in a histidyl-tRNA synthetase gene PCR. All samples were amplified and all products from PMNL DNA showed an equivalent PCR signal or more when compared to a paired adherent PBMC PCR product, except the sample in track 17 where a diffuse band is seen (Figure 3.11).

3.5 DISCUSSION

Pure populations of cells (> than 90% in all cases) were amplified with highly sensitive and thoroughly optimized PCRs. The level of detection of these PCR systems was as little as 1-10 copies of an IE1-containing plasmid. Targets were amplified from multiple clinical isolates and from adherent PBMC of all healthy seropositive individuals tested, and hybridized specifically to corresponding HCMV probes. In addition, in every case where HCMV PCR was not positive it was shown using a control PCR for a housekeeping gene (histidyl tRNA synthetase) that this was not due to a general inability to amplify DNA. Consequently, these PCR systems were sensitive and specific, and allowed a detailed analysis of the sites of low copy persistence of HCMV.

Employing the above techniques, HCMV DNA was found primarily in CD3- or non-T cells of PBMC from healthy seropositive carriers. Further analysis of both adherent cells and CD14 positive cells (representing mature monocytes in both cases) suggested that the source of the HCMV probe-positive signal in the CD3- cells was primarily monocytes. In addition, 1 healthy seropositive subject, who had already been shown to have a weak positive HCMV signal in the FACS sorted CD3+ cells, showed evidence of HCMV DNA in the CD8+ T cell population

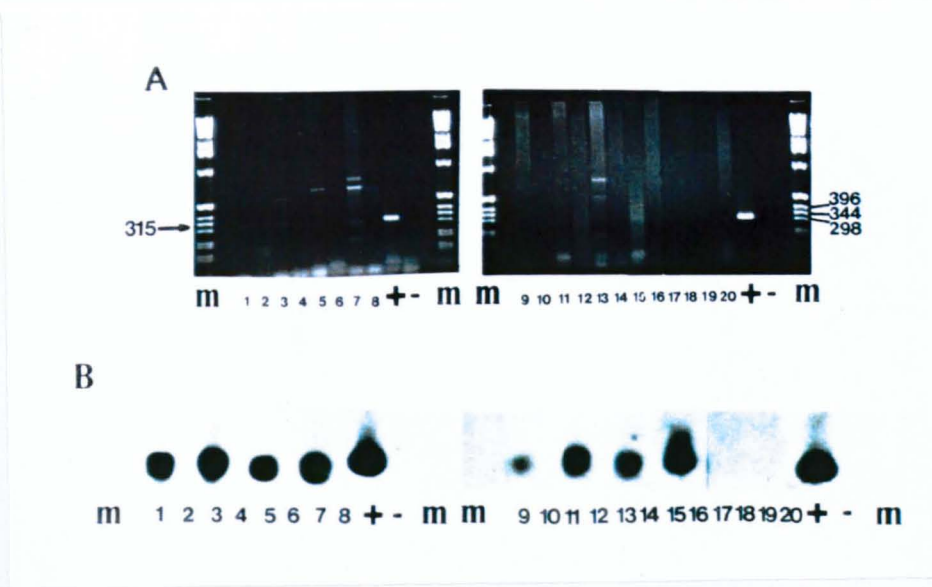


Figure 3.10. HCMV PCR Analysis of PMNL of Healthy Carriers. (A) Ethidium bromide-stained agarose gel of the HCMV PCR products are shown. M is a 1 kb ladder (Gibco BRL). The 315 bp amplification product is marked with an arrow. The (+) column represents amplification of HCMV AD169; while the column marked (-) is the non-DNA containing negative control. Tracks 1 & 2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, 11 & 12 and 13 & 14 are paired samples from 7 seropositive individuals. Tracks 1,3,5,7,9,11 and 13 are amplification products from PBMC DNA and 2,4,6,8,10,12 and 14 are amplification products from PMNL DNA. Tracks 15 and 16 are the amplification products of the PBMC and PMNL respectively for the one seronegative/PCR positive subject. Tracks 17, 18, 19 and 20 are paired samples from 2 seronegative/PCR negative individuals with tracks 17 and 19 amplification from PBMC and 18 and 20 from PMNL. Panel B: The Southern blot from the gel shown in (A) after probing with an internal oligonucleotide to the PCR product is shown. Identical numbering as in (A) is used.

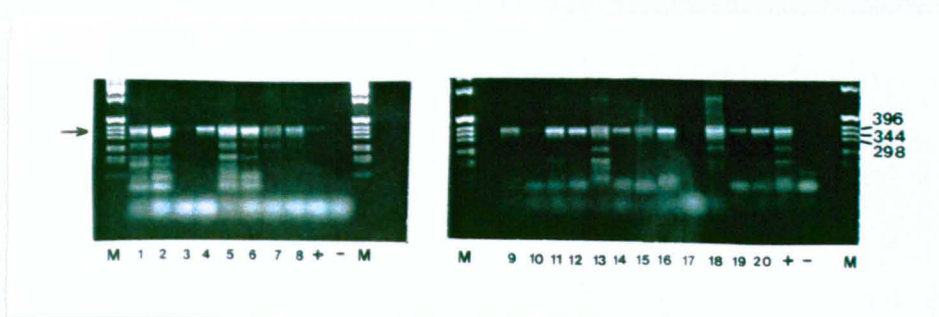


Figure 3.11. Control Gene (Histidyl-tRNA Synthetase) PCR of PMNL DNA. Ethidium bromide stained agarose gel of the histidyl-tRNA synthetase control PCR. A 1 kb ladder is used as a DNA size marker and is denoted M. Tracks 1 & 2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, 11 & 12; 13 & 14, 15 & 16, 17 & 18 and 19 & 20 are paired samples from the 10 subjects analyzed in Figure 3.10. Tracks 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 are amplification products from PBMC DNA and 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 are amplification products from PMNL DNA. The (+) column is 100 ng of human DNA and the (-) column is a non-DNA containing reaction mix negative control. Although many smaller bands due to mis-priming can be seen, the 370 bp predicted amplification product is clearly seen in all lanes except the negative control (-) and lane 17 which shows a diffuse band.

albeit to a lesser degree than seen in monocyte-enriched populations. The results presented here are in contrast to those of Schrier *et al.* (1985), in which T cells were suggested as being the major site of persistence by in situ hybridization.

Another report (Toorkey & Carrigan, 1989) using an anti-HCMV IE1 antibody and a histochemical detection method, showed a wide cell type and organ distribution of HCMV. Although monocytes were not reported as a cell type showing positive signal several tissue macrophages were positive including liver Kupffer cells, alveolar macrophages and splenic macrophages. Our results would be consistent with the macrophage sites identified, as monocytes differentiate into tissue macrophages (Thomas *et al.*, 1976; Gale *et al.*, 1978; Gordon *et al.*, 1988; Johnston, 1988). It is, however, difficult to determine how closely these data reflect the healthy state, as samples were obtained from autopsy specimens of previously healthy individuals who succumbed to acute traumatic deaths.

My examination, however, analyzed specific cell populations from healthy carriers and clearly showed that primarily monocytes, and to a lesser degree CD8+ lymphocytes in some subjects, are sites of HCMV persistence in PBMC of healthy carriers. It is recognized that the analysis of T cell sites of persistence, presented here, represents only 1 individual who was selected on the basis that a weak HCMV signal was seen in the CD3+ cell sorted population. This limited data and biased selection preclude drawing definitive conclusions on the extent to which T cells may harbor HCMV in healthy carriers.

In addition, my study showed that approximately 30% of seronegative subjects analyzed had evidence of HCMV probe positive signals in adherent PBMC. This frequency of seronegative HCMV carriers is in agreement with the published literature (Stanier *et al.*, 1989; Bevan *et al.*, 1991; Stanier *et al.*, 1992). In 1 of these subjects, the

predominant HCMV-specific signal was found in the CD3- population of FACS-separated cells, similar to that seen in the FACS-separated cell populations from seropositive subjects. It is doubtful, that these results represent contamination as exhaustive attempts were made to prevent this occurrence. These subjects most likely, represent a group of individuals whose anti-HCMV antibody levels are below the level of detection of the serologic assays used. In addition, reports of other PCR positive but seronegative or antigen negative viral carriers have been published for HIV and hepatitis B virus (Imagawa *et al.*, 1989; Wang *et al.*, 1990). Finally, although this implies that HCMV infection may be more widespread than conventional seroepidemiology suggests (Bevan *et al.*, 1991) it is only rarely reported that seronegative subjects harbor transmissible virus (Bowden, 1991). It is much more commonly reported that immunocompromised adults and neonates receiving HCMV seronegative blood products show no evidence of HCMV transmission (Yeager *et al.*, 1981; Adler *et al.*, 1983; Bowden *et al.*, 1986; Chou, 1986; Paya *et al.*, 1989). Rare cases of HCMV transmission seen with seronegative blood products despite the fact that as many as 30% may come from seronegative HCMV carriers may be explained by these blood products containing less than a minimal infectious dose. This is supported by the fact that known HCMV seropositive blood components only transmit the virus to susceptible recipients in less than 1/3 of all cases. In fact, when analyzing premature neonates (one of the most susceptible populations) who received blood from at least one positive donor, only 24 % of the infants acquired HCMV infection in 2 separate studies (Yeager *et al.*, 1981; Adler, 1983). Similarly, increased HCMV transmission can be correlated with increased amounts of blood products transfused (Wilhelm *et al.*, 1986).

The data presented here, suggested that monocytes are a major site of persistence in peripheral blood (Taylor-Wiedeman *et al.*, 1991; Taylor-Wiedeman *et al.*, in press). However, as it is difficult to infect freshly isolated monocytes with HCMV and PBMC appear not to replicate virus (Rice *et al.*, 1984; Einhorn & Öst, 1984), the issue arises as to how these cells may acquire and maintain persistent HCMV. It is known that bone marrow progenitors can be infected *in vitro* (Sing & Ruscetti, 1990); therefore, one explanation of monocytic cell persistence may be that the cells acquire HCMV at an early stage of their differentiation in the bone marrow. However, the same myeloid precursors give rise to both monocytes and PMNL (Metcalf, 1971; Clark & Kamen, 1987), but PMNL do not appear to harbor HCMV in healthy carriers whilst monocytes do. Therefore, if early myeloid precursors are targets for HCMV infection it would appear that differentiation results in a partitioning of HCMV to the monocyte fraction. One explanation for this could be that HCMV infected PMNL precursors may be unresponsive to colony stimulating growth factors resulting in a loss of these cells (Sing & Ruscetti, 1990).

Ultimately, it is well documented that HCMV can be detected in PMNL of immunocompromised patients and in rare instances in immunocompetent subjects who are symptomatic with HCMV infection (Fiala *et al.*, 1975; Rinaldo *et al.*, 1977; Saltzman *et al.*, 1988; Dankner *et al.*, 1990, Gerna *et al.*, 1991; Revello *et al.*, 1992). Common to all these cases, however, is viremia usually with serious active disease. Nevertheless, in one report where 30 HCMV seropositive/HIV infected patients with no HIV disease and no overt HCMV disease were analyzed, no HCMV DNA could be detected in PMNL enriched cells (Gerna *et al.*, 1990). Whilst, these patients may be similar to the normal HCMV carrier, the PCR sensitivity was not adjusted to detect low copy DNA and therefore, the lack

of sensitivity precludes drawing definitive conclusions concerning normal subjects.

Some reports have shown evidence of IE, E, or late HCMV gene expression in PMNL of viremic patients either by in situ hybridization (Dankner *et al.*, 1990) or by immunofluorescent detection of nuclear localized IE proteins and in rare instances even late HCMV antigens (Revello *et al.*, 1992). It is, however, not clear whether this detection of HCMV gene expression might result from phagocytosis of intact viral particles with subsequent gene expression or might be due to direct infection of PMNL cells (Dankner *et al.*, 1990, Revello *et al.*, 1992). Nonetheless, results presented above would suggest that HCMV is not normally present in PMNL of aviremic healthy HCMV carriers.

CHAPTER 4

ENDOGENOUS HCMV GENE EXPRESSION IN ADHERENT MONOCYTES FROM HEALTHY SEROPOSITIVE CARRIERS

4.1 INTRODUCTION

In chapter 3, investigation of sites of persistence of HCMV in the peripheral blood implicated monocytes as a major site of HCMV persistence. Consequently, experiments were designed to analyze primary monocytes for evidence of HCMV transcription during viral persistence. In particular, the spectrum of HCMV gene expression in the asymptomatic healthy carrier was analyzed to determine if viral persistence occurred by low level replication or by restricted gene expression as is seen with HSV, VZV and EBV (Stevens *et al.*, 1989; Garcia-Blanco & Cullen, 1991).

Previously, Schrier *et al.* (1985), reported finding HCMV IE transcripts in lymphocytes from peripheral blood of healthy carriers by in situ hybridization, but were unable to coculture virus. The same group, however, (Gnann *et al.*, 1988) was unable to detect IE or late transcripts in seropositive donor kidney biopsies obtained pre-transplant even though lymphocytes would have been present. Disparity in results from these two reports suggested that in situ hybridization may not be sensitive enough to detect rare copy number transcripts.

Toorkey and Carrigan (1989), have also reported detection of IE1 proteins in multiple tissues and cell types from post mortem examinations. In this report, IE1 proteins but not late proteins were detected using a histochemical technique, the sensitivity of which was not determined. In addition, it is difficult to know what changes may have occurred in these tissues after death. It is well known that environmental

stresses induce a typical physiological reaction, the heat-shock response (Ashburner *et al.*, 1979), which has been shown to partially activate HCMV in semipermissive cells (Zerbini *et al.*, 1986; Zerbini *et al.*, 1985). It is possible, therefore, that the IE1 expression seen in the autopsy samples was a reflection of this enhanced viral activity in response to post mortem changes and not a true reflection of gene expression seen in the healthy carrier.

Thus, in an attempt to examine gene expression during HCMV persistence in healthy carriers, peripheral blood monocytes were obtained directly from seropositive healthy subjects and examined with one of the most sensitive techniques available, reverse transcription followed by PCR (Saiki *et al.*, 1985; Yang *et al.*, 1989; Wang *et al.*, 1989; Chelly *et al.*, 1990; Holodniy *et al.*, 1991). The transcription units examined were IE1 and IE2 (Akrigg *et al.*, 1985; Stenberg *et al.*, 1985), the HQLF-1 immediate early gene (Weston, 1988), and a true late gene (pp28) located at 0.63 to 0.65 map units in the UL coding region (Meyer *et al.*, 1988).

4.2 MATERIALS AND METHODS

General materials and methods are discussed in Chapter 2 including a detailed description of the control PCR, histidyl-tRNA synthetase, in section 2.11.5.

Where possible, intron-spanning PCRs were used to analyze the cDNA products of reverse transcription resulting in a size difference between DNA and cDNA amplification products. Experiments analyzing gene expression, examined a minimum of three healthy seropositive subjects and were performed at least twice to confirm reproducibility.

4.2.1 First Strand Reverse Transcription

First strand reverse transcription was carried out using 10 µg of total RNA (approximately equivalent to 10^5 cells) in a 50 µl reaction in the presence of 10 µg/ml of oligo dT primer. Non-oligo dT primed RNA samples served as a control to determine the presence of any contaminating DNA or cDNA by PCR. Initially, oligo dT or H₂O was added to 10 µg of RNA and incubated at 68 °C for 5 minutes, cooled to 20 °C over 10 minutes and held for a further 5 minutes at 20 °C. Samples were reverse transcribed in 50 µl containing: 50 mM KCl, 50 mM Tris-HCl (pH 8.3 at 42 °C), 6 mM MgCl₂, 5 mM spermidine, 1 mM each dNTPs, 1 U rRNasin (Promega), 4 mM DTT, and 4 U RT (superRT: HT Biotechnology, UK), by incubating for 1 hour at 42 °C, then 8 minutes at 95°C. Samples were then cooled to 4 °C and held at -70 °C until PCR. All incubations were performed in a Perkin Elmer Cetus Thermocycler using GeneAmp™ (Perkin Elmer Cetus) 0.5 ml tubes.

4.2.2 Nested Intron-Spanning IE1 PCR

The design of the nested intron-spanning PCR for IE1 as well as the external, internal, and probe oligonucleotide sequences and their locations are detailed in Figure 4.1. For this PCR, 1/5 of the reverse transcription (i.e. reverse transcribed RNA from approximately 2×10^4 cells) was amplified in a 40 µl reaction mix (50 mM KCl, 10 mM Tris-HCl pH 8.5 at room temperature, the primary PCR used 1 mM MgCl₂ and the secondary PCR used 1.5 mM MgCl₂, 0.01% gelatin, 0.5% Tween 20, 0.1% Triton X-100, 1 mM of each primer, 200 mM of each dNTP, and 1 U of Promega *Taq* polymerase). The nested PCR used 2 µl of the primary PCR mix as template. For both the primary and nested PCRs, a modified hot start (section 3.3.1) was used and was followed by an initial denaturation of 94

°C for 5 minutes then 50 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 90 seconds.

4.2.3 Nested Intron-Spanning IE2 PCR

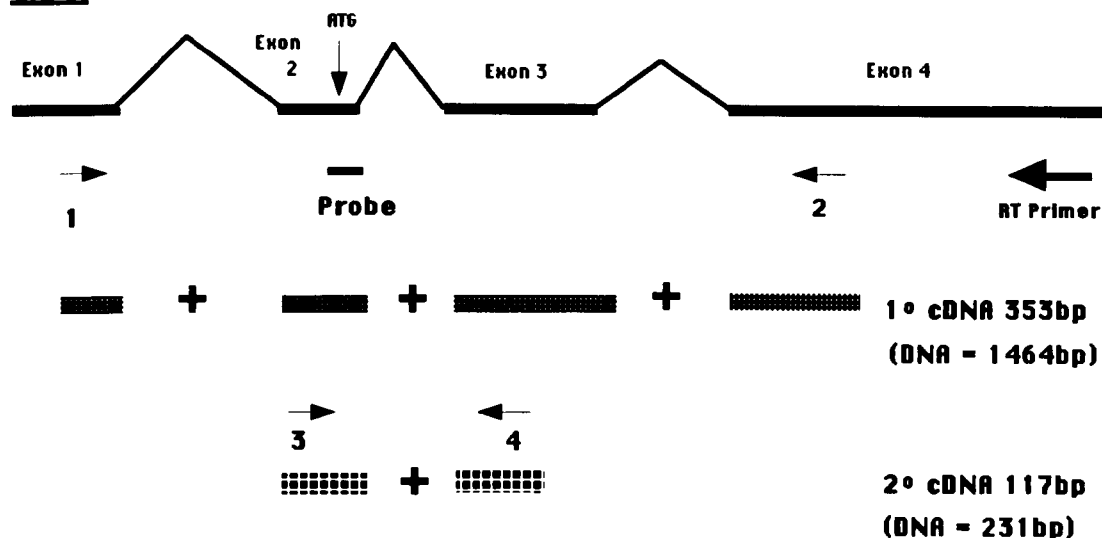
The HCMV IE2 PCR design as well as the internal, external, probe oligonucleotides and their locations are shown in Figure 4.2. The optimized reaction mix and the cycling parameters were found to be the same as described above for the nested intron-spanning HCMV IE1 PCR with the following exception: both the primary PCR and the secondary PCR required 0.25 mM spermidine (Grausz, 1991).

4.2.4 Nested Intron-Spanning HQLF-1 PCR

The design of the HQLF-1 IE gene PCR as well as the sequence of the primers and probe used in this analysis are shown in Figure 4.3. For this PCR, 1/5 of the reverse transcription (i.e. reverse transcribed RNA from approximately 2×10^4 cells) was amplified in a 40 μ l reaction mix (50 mM KCl, 10 mM Tris-HCl pH 8.5 at room temperature, 1 mM MgCl₂, 0.01% gelatin, 0.5% Tween 20, 0.1% Triton X-100, 1 mM of each primer, 200 mM of each dNTP, and 1 U of Promega *Taq* polymerase). The nested PCR used 2 μ l of the primary PCR mix as template. For both the primary and nested PCRs, a modified hot start (section 3.3.1) was used and was followed by 94 °C for 5 minutes of initial denaturation then 50 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 90 seconds.

4.2.5 Nested Late Gene PCR

The late PCR amplified a portion of the major 28 kd structural phosphoprotein gene (Meyer *et al.*, 1988), and, here after, is termed the late HCMV PCR. It was a nested but non-intron-spanning PCR as this is an unspliced gene. The optimized conditions were identical to those described

4.1A**4.1B**

Sense primer: 5' > GGTGCATTGGAACGCGGATT < 3'

Anti-sense primer: 5' > ATTCTATGCCGCACCATGTCCA < 3'

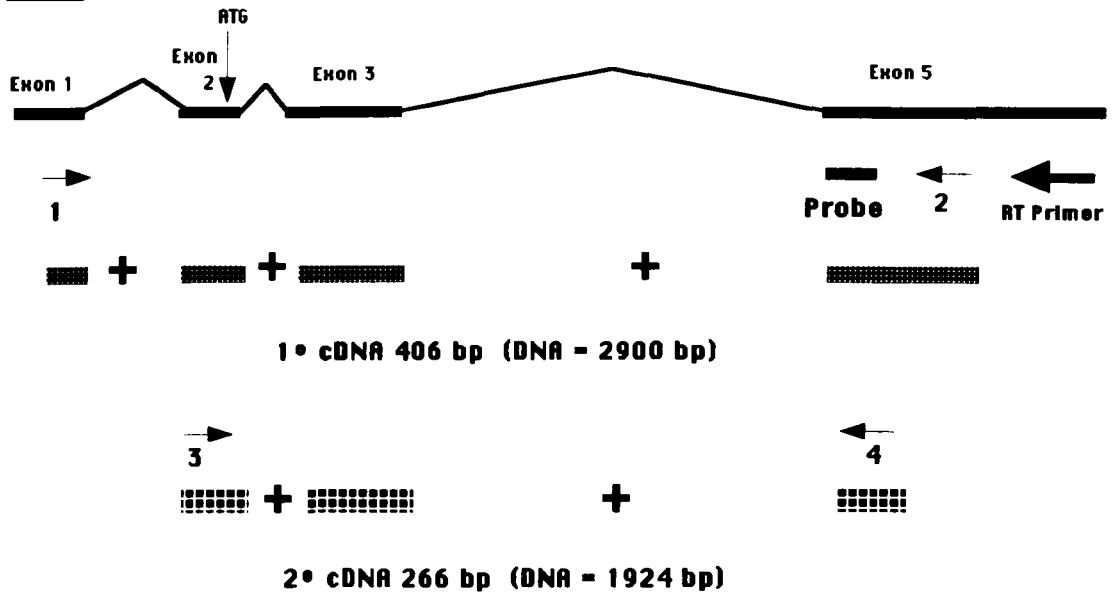
Nested sense primer: 5' > ATGGAGTCCTCTGCCAAGAG < 3'

Nested anti-sense primer: 5' > CATAGTCTGCAGGAACGTCGT < 3'

Probe: 5' > GACCCTGATAATCCTGACGA < 3'

Figure 4.1. HCMV IE1 Nested Intron-spanning PCR. In panel A the organization of the HCMV IE1 region is shown at the top and the design of the nested intron-spanning PCR is shown at the bottom. The external primers (arrow 1 = sense primer and arrow 2 = anti-sense primer) are shown in exon 1 and 4, respectively. Whereas, the internal sense primer (arrow 3) was in exon 2 and the anti-sense primer (arrow 4) was in exon 3. The external primer pair yielded a DNA product of 1464 bp and a cDNA product of 353 bp. The internal primers yielded a DNA product of 231 bp and a cDNA of 117 bp. The oligonucleotide probe was also present in exon 2, internal to primer # 3. The reverse transcription oligo dT primer is labeled RT primer. Sequences of PCR primers and probe are shown in panel B.

4.2A



4.2B

Sense primer: 5' > GGTGCATTGGAACGCGGATT < 3'

Anti-sense primer: 5' > GCGCTGCTAACGCTGCAAGAG < 3'

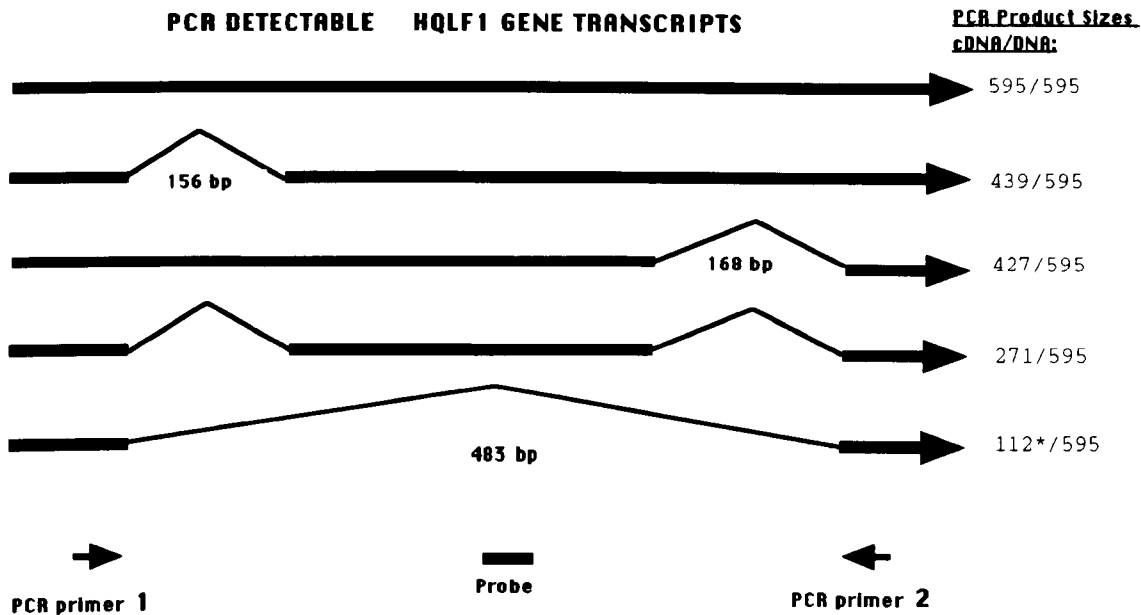
Nested sense primer: 5' > GACCCTGATAATCCTGACGA < 3'

Nested anti-sense primer: 5' > ATACCGGCATGATTGACAGCCT < 3'

Probe: 5' > GACATCCTCGCCCAGGCT < 3'

Figure 4.2. HCMV IE2 Nested Intron-spanning PCR. Panel A shows the organization of the HCMV IE2 region (top) and the design of the nested intron-spanning IE2 PCR (bottom). Panel B shows the sequences of the primers and probe. The external primers yielded a DNA product of approximately 2900 bp and a cDNA of 406 bp, the upstream primer (arrow 1) was in exon 1 and the downstream anti-sense primer (arrow 2) was in exon 5. Whereas, the internal primers yielded a DNA product of approximately 1924 bp and a cDNA of 266 bp. These sense (arrow 3) and anti-sense (arrow 4) primers were located in exon 2 and 5, respectively. The probe is specific for exon 5.

4.3A



4.3B

sense primer: 5' > ATCCTGGCCGTCCTTGTTCCCT < 3'

Anti-sense primer: 5' > GTTAGAGTCCGTACCTTGCA < 3'

Probe: 5' > TGAGCAGTACCACCATGATG < 3'

Figure 4.3. Design of HQLF-1 PCR. Five predicted products from transcription of the HQLF-1 gene are shown (Weston, 1988). The location of the single primer pair used to detect cDNA and DNA from the HQLF-1 open reading frame and the size of the predicted products are detailed in 4.3 A. The sequences of the primers and probe, the latter of which would recognize all but the smallest cDNA product (*) are shown in 4.3 B.

for the intron-spanning IE1 PCR above. The external primer pair amplified a 548 bp product and the internal primers amplified a 389 bp product. The primers amplified an area of the HCMV genome which has no complementarity to that of other herpesviruses or human DNA. These primers were used successfully to amplify HCMV DNA from 5 unrelated clinical HCMV isolates and 10 healthy subjects. The sequence of the primers and probe are as follows:

Sense primer: 5' > GAGGATGACGATAACGAGGA < 3'

Anti-sense primer: 5' > TCAAACAGCACATTAGACACACGG < 3'

Nested sense primer: 5' > GACAGTAGTAGCGGCAGCCA < 3'

Nested anti-sense primer: 5' > GACTCGCGAATCGTACGCGA < 3'

Probe: 5' > GACCTAGACGAAGAGGACACCTCAAT < 3'

4.3 RESULTS: TRANSCRIPTIONAL ANALYSIS OF PRIMARY MONOCYTES IN THE HEALTHY ASYMPTOMATIC CARRIER

4.3.1 Sensitivity of the Reverse Transcription

The sensitivity of the reverse transcription allowed for detectable message from the equivalent of 1 infected cell when the IE1 PCR was used for amplification and is detailed in Figure 4.4. The level of detection, however, was ultimately dependent on the sensitivity of the individual PCRs and is detailed in the results section 4.3.3.

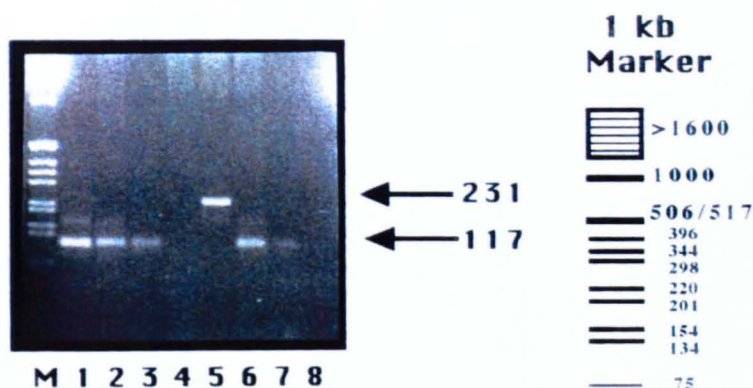
4.3.2 Sensitivity of the PCRs

The sensitivity of the IE1 nested PCR (Figure 4.5) was estimated to be equivalent to 1 copy of linearized cDNA. By comparison to known plasmid copy number, 1 femtogram of a standard HCMV infected cell DNA stock was considered to be approximately equivalent to 1 copy (compare intensity of lanes 4 and 7 in Figure 4.5)

The IE2 nested PCR, the HQLF-1 gene PCR and the late HCMV gene PCR were able to detect 10, 10, and 100 femtograms, respectively, of the stock infected cell DNA which was the same stock used above and for all PCRs described in this study (Figures 4.6 and 4.7). Therefore, by extrapolation from estimations made with the IE1 PCR DNA and known copy number plasmid amplifications (Figure 4.5), the estimated sensitivities of the IE2, HQLF-1 and late PCR were 10, 10, and 100 copies of HCMV, respectively,

All primers amplified an area of the HCMV genome which has no complementarity to human DNA or other herpes viruses (Shaw *et al.*, 1985; Chee *et al.*, 1990).

A



B

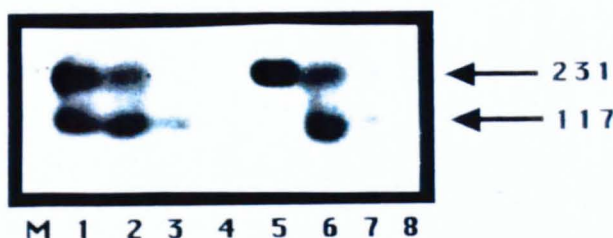


Figure 4.4. Sensitivity of the Reverse Transcription. Panel A shows the ethidium bromide gel of the intron-spanning IE1 nested PCR (detailed in Figure 4.1) which amplified cDNA templates after reverse transcription of RNA from HCMV AD169 infected fibroblasts. Specific cDNA bands are marked with the 117 bp arrow and DNA bands are marked with the 231 bp arrow. Lanes 1 through 4 are RT-PCR of 10^4 infected cells, 10^2 infected cells, 1 infected cell, and a H₂O RT negative control, respectively. Lanes 5 through 8 are PCR controls and detailed as follows: Lane 5 is 100 femtograms (100 copies) of DNA only, while Lanes 6 through 8 are 10 copies of linearized pGEM-2 IE1 cDNA plasmid along with 10 femtograms of infected cell DNA, 1 copy of linearized pGEM-2 IE1 cDNA plasmid along with 1 femtogram of infected cell DNA, and a H₂O PCR control. The DNA marker (lane M) is a 1 kb ladder (Gibco) with the band sizes detailed at the top right. Panel B shows the Southern blot of the gel from (A). Specific probe positive bands are seen in lanes 1, 2, 3, 5, 6, & 7 while H₂O controls (lanes 4 & 8) remain probe negative. For the RT-PCR samples in lanes 1, 2, & 3, both DNA and cDNA were present in the initial template (RNA was not treated with DNase) therefore both bands are seen on Southern blot and a DNA amplification product also can be seen in lane 1 on the ethidium bromide gel.

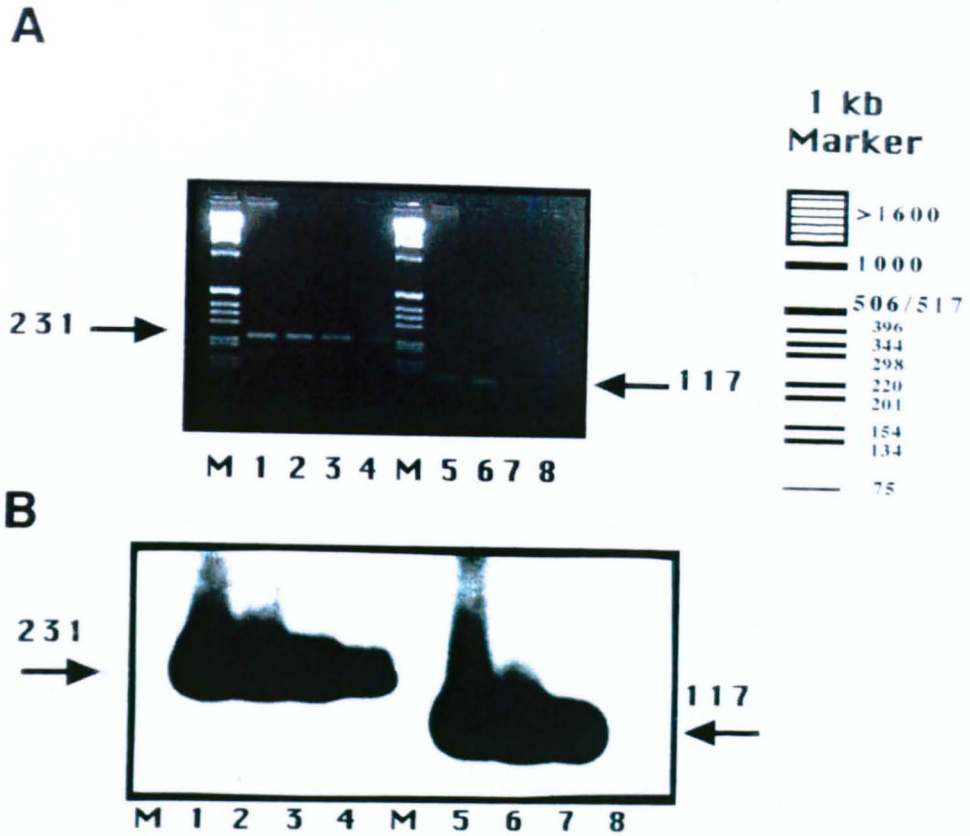


Figure 4.5. Sensitivity of the IE1 Nested Intron-Spanning PCR. Panel A shows the ethidium bromide stained gel of the comparison of dilutions of DNA from a standard stock of HCMV AD169 infected fibroblasts (lanes 1-4) and linearized pGEM-2 IE1 cDNA plasmid (lanes 5-7). The size of amplification products are marked: 231 bp arrow for DNA and 117 bp arrow for cDNA. Lanes 1 through 4 show: 100 femtograms of infected cell DNA, 50 femtograms of infected cell DNA, 10 femtograms of infected cell DNA and 1 femtogram of infected cell DNA, respectively. Lanes 5 through 8 are amplifications of 10^2 copies of pGEM-2 IE1, 10^1 copies of pGEM-2 IE1, 1 copy of pGEM-2 IE1, and a H₂O only negative control, respectively. Lanes M are the 1 kb marker, detailed at the right of panel A. Panel B shows the Southern blot of panel A. HCMV probe positive bands are seen in lanes 1,2,3,4,5,6,7, while the H₂O control remained probe negative.

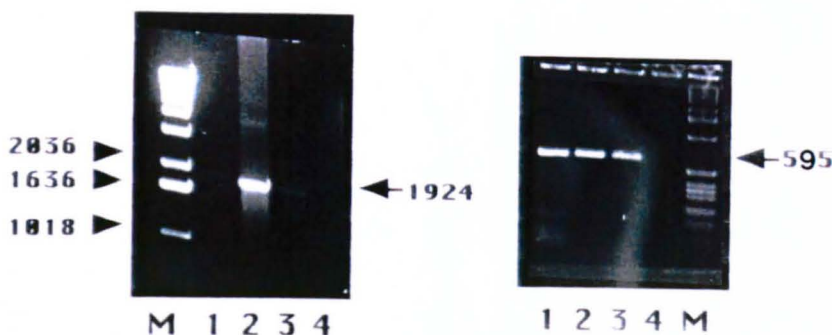


Figure 4.6. Sensitivity of the IE2 Nested Intron-Spanning PCR and the HQLF-1 PCR. Both ethidium bromide gels show the amplification of the standard HCMV infected cell DNA stock used as a control for all PCRs. The gel on the left shows the specific 1924 bp products after the IE2 PCR in lane 1 (10 picograms of infected cell DNA after a 50 cycle non-nested amplification using the internal primers only i.e. without prior amplification using the external primers), lane 2, 3, and 4 (100, 10, and 1 femtogram of infected cell DNA after a fully nested IE2 PCR). In addition, as the IE2 DNA products are much larger than most used in this analysis the specific sizes of the larger marker bands (1018, 1636, and 2036 bp) for the 1 kb marker (lanes M) are noted on the left of the IE2 gel. Thus, for the IE2 PCR it can be seen that the limit of detection on ethidium bromide gel is 10 femtograms (10 copies, see Figure 4.5) of the standard stock of infected cell DNA. The gel on the right shows the specific 595 bp DNA product from the HQLF-1 gene PCR amplification. Lanes 1-4 represent: 1000, 100, 10, and 1 femtogram of the standard HCMV infected cell DNA stock. It can be seen that the limit of detection on an ethidium bromide gel is 10 femtograms (10 copies) of infected cell DNA.



Figure 4.7. Sensitivity of the Late Gene PCR. The late gene PCR products after a fully nested amplification are shown. Lanes 1-4 are as follows: amplification of 10,000 femtograms, 1000 femtograms, 100 femtograms and 10 femtograms of the standard stock of HCMV infected cell DNA. Thus, the limit of detection for this PCR was only 100 copies of HCMV (lane 3). In addition, this PCR showed less distinct bands on the ethidium bromide gel at higher template dilutions. This was the only PCR in this analysis where greater template quantity lead to less distinct amplification products after nested PCR. However, as seen below, the Southern blot clearly defines the presence of the late amplification products in lanes 1-3 while the other less distinct bands running higher in the gel in lanes 1 and 2 are not probe positive and therefore likely due to mispriming events. Lane M is a 1 kb marker (Gibco).

4.3.3 Gene Expression in Adherent Monocytes

Comprehensive RT-PCR analysis to detect IE1 (Figure 4.8), IE2 (Figure 4.9), HQLF-1 (Figure 4.10) and late gene (Figure 4.11) transcripts showed no evidence of these RNA in primary monocytes. Nevertheless, after reverse transcription, primary monocyte RNA were successfully amplified in a control gene RT-PCR, histidyl-tRNA synthetase (Figure 4.12), and HCMV DNA was shown to be present by extracting DNA separately from the same cells used for RNA extraction (Figure 4.13).

In all RT-PCRs, RNA from primary monocytes were reverse transcribed and amplified along with matched controls which consisted of RNA and all other components of the RT mix except the RT-primer, oligo dT (denoted RT sample controls). These RT-PCR controls were necessary to detect the presence of DNA, which was important where non-intron-spanning PCR was used (late gene PCR), and also controlled for any cDNA contamination of the individual RNA samples. The RT H₂O control was a complete RT mix with H₂O instead of RNA and controlled for cDNA contamination of all RT components. RT-PCR of RNA, from a standard lot of HCMV infected cells, was used to assess RT-PCR sensitivity. Results are as follows: the IE1 RT-PCR was able to detect the equivalent of 1 infected cell (Figure 4.4 and Figure 4.8) while the IE2 RT-PCR (Figure 4.9, lanes 8 & 9), the HQLF-1 RT-PCR (Figure 4.10, lanes 4 & 5) and the late gene RT-PCR (Figure 4.11, lanes 17, 18 & 19) were able to detect the equivalent of 100 infected cells but not 1 infected cell. The standard RNA stock from HCMV infected cells was found to contain some HCMV DNA (Figure 4.8, lanes 7 & 8, Figure 4.9, lanes 7 & 8 and Figure 4.12, lane 7) after acid phenol RNA extraction. All RNA isolated from individual healthy carriers underwent RNase-free DNase treatment (section 2.8.2), however, it only became necessary to DNase treat the HCMV infected cell RNA control when non-intron-spanning PCR was used (late gene PCR

Figure 4.11, lanes 17, 18 & 19). Finally, it should be noted that some of the PCR amplifications yielded small non-specific bands (for example Figure 4.10, lanes 7 and 8) seen on the ethidium bromide gels which were not probe positive and most likely due to mispriming events. Several small misprimed products were seen in the IE2 PCR but the smallest band (< 75 bp DNA marker) seen in every lane was most likely oligonucleotide PCR primers. It is, however, clearly seen that no probe-positive bands were present in any of the subjects samples while positive controls (both RT and PCR) amplified probe-positive products of the appropriate size and negative controls (both RT and PCR) yielded no probe-positive products.

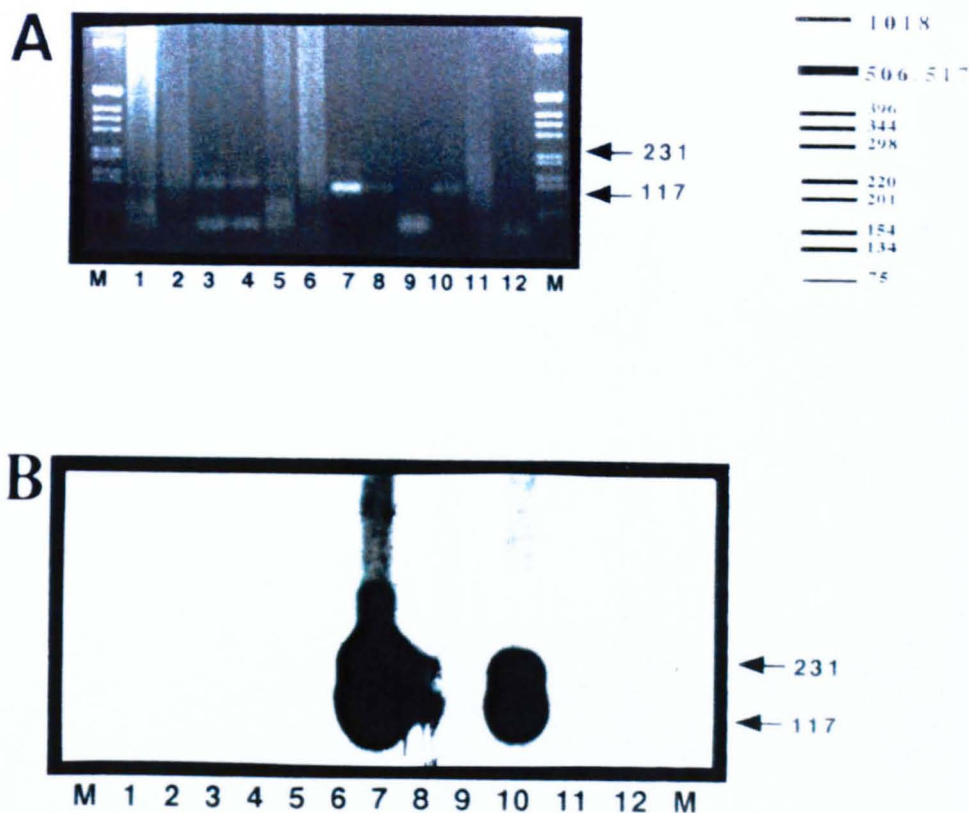


Figure 4.8. IE1 RT-PCR of Primary Monocytes. The top panel (A) shows the ethidium bromide gel of the intron-spanning IE1 nested RT-PCR on the left and a detailed description of the 1 kb DNA marker (lanes M) on the right. Specific sizes of the predicted products are marked with an arrow and represent DNA = 231 bp and cDNA = 117 bp. Lanes 1 & 2, 3 & 4, and 5 & 6, are paired RT-PCR samples of primary monocytes from three healthy subjects. Lanes 1, 3, and 5 contain complete RT-PCR mixes and lanes 2, 4, and 6 are RT sample controls, respectively. Other lanes are as follows: lanes 7 & 8 (RT-PCR of 100 and 1 HCMV infected cells, respectively), lane 9 (a H₂O RT control), lane 10 (a PCR control containing 10 copies of IE1 cDNA and 10 copies of DNA), lanes 11 and 12 (PCR H₂O controls). The bottom panel (B) shows the Southern blot of the ethidium bromide gel. Probe positive bands can be seen in lanes 7, 8, and 10 only, the RT and PCR positive controls.

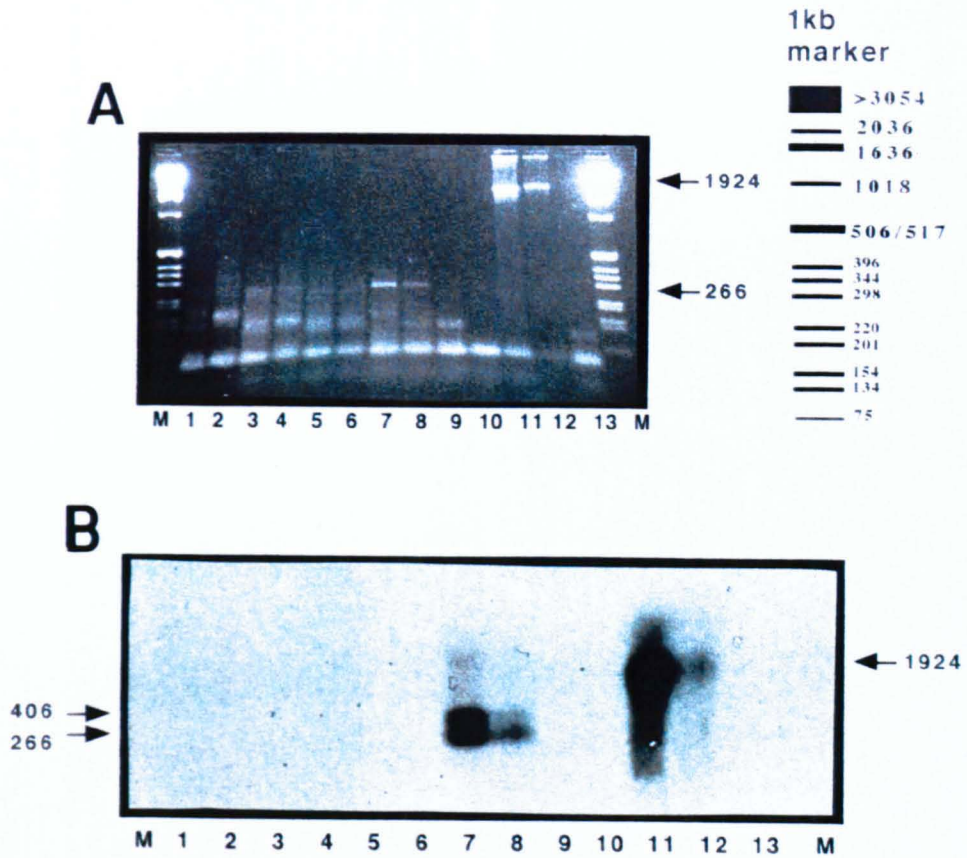


Figure 4.9. IE2 RT-PCR of Primary Monocytes. The top panel (A) shows the ethidium bromide gel of the intron-spanning IE2 nested RT-PCR on the left and a detailed description of the 1 kb DNA marker (lanes M) on the right. Specific sizes of the predicted products are marked with an arrow and represent DNA = 1924 bp and cDNA = 406 bp (primary amplification) or 266 bp (nested amplification). Lanes 1 & 2, 3 & 4 and 5 & 6, are paired RT-PCR samples of primary monocytes from three healthy subjects. Lanes 1, 3, and 5 contain complete RT-PCR mixes and lanes 2, 4, and 6 are RT-PCR sample controls, respectively. Other lanes are as follows: lanes 7, 8, & 9 (RT-PCR of 1000, 100 and 1 HCMV infected cells, respectively), lane 10 (a H₂O RT control), lanes 11 & 12 (PCR control containing 100 and 10 copies of DNA, respectively) and lane 13 (PCR H₂O control). The bottom panel (B) shows the Southern blot of the ethidium bromide gel using an exon 5 specific probe. Probe positive bands are seen in lanes 7 & 8 and 11 & 12, the RT and PCR positive controls, respectively, In lanes 7 and 8 while the 266 bp nested cDNA product is clearly seen, the primary cDNA product, 406 bp, can also be seen (lane 7 > lane 8).

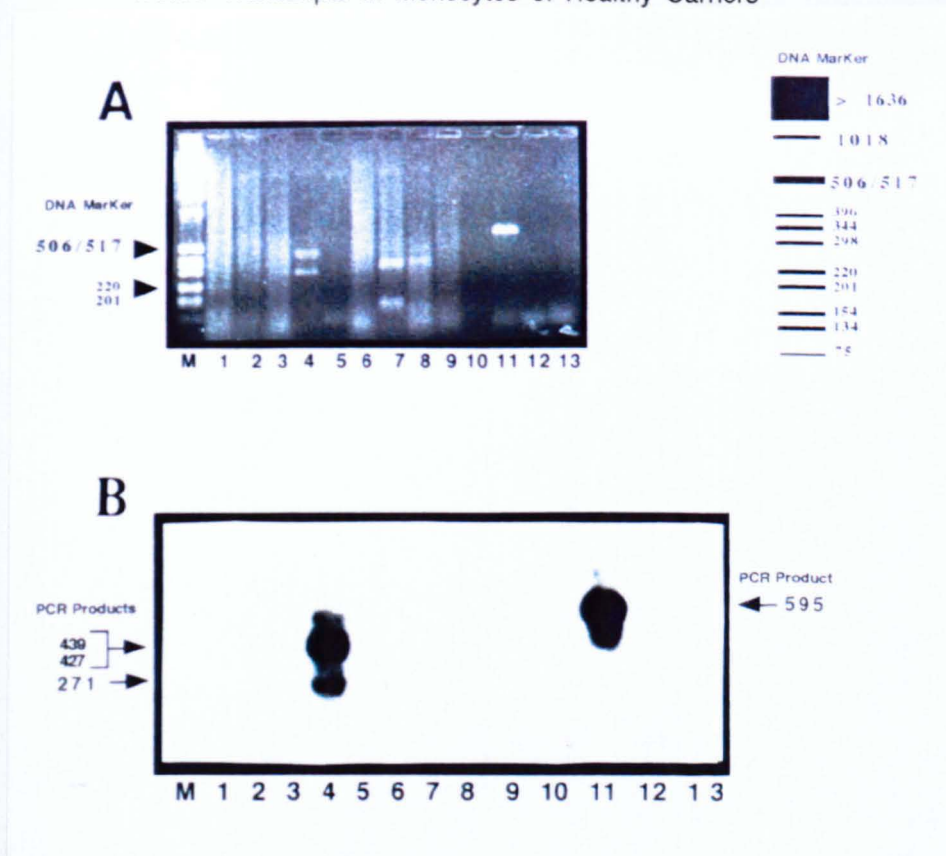


Figure 4.10. HQLF-1 RT-PCR of Primary Monocytes. The top panel (A) shows the ethidium bromide gel of the HQLF-1 RT-PCR on the left and on the right is a detailed description of the 1 kb DNA marker (lanes M). Lanes 1 & 6, 2 & 7 and 3 & 8 represent paired RT-PCR samples of primary monocytes from three healthy subjects. Lanes 1, 2, and 3 contain complete RT-PCR mixes and lanes 6, 7, and 8 are RT sample controls, respectively. Lane 4 shows the positive cDNA products of 439, 427, and 271 bp in size. Other lanes are as follows: lanes 4 & 5 (RT-PCR of 100 and 1 HCMV infected cells, respectively), lanes 9 & 10 (H₂O RT controls), lanes 11 & 12 (PCR control containing 10 and 1 copy of DNA, respectively) and lane 13 (PCR H₂O control). The bottom panel (B) shows the Southern blot of the ethidium bromide gel. Specific sizes of the predicted products are marked with an arrow on the Southern blot and represent DNA = 595 bp and cDNA = 439, 427, and 271 bp. The smallest detectable product, 112 bp (Figure 4.3) was not seen on the ethidium bromide gel and would not have been detectable on the Southern blot with the internal oligonucleotide probe that was used. Probe positive bands can be seen in lanes 4 and 11, the RT and PCR positive controls, respectively.

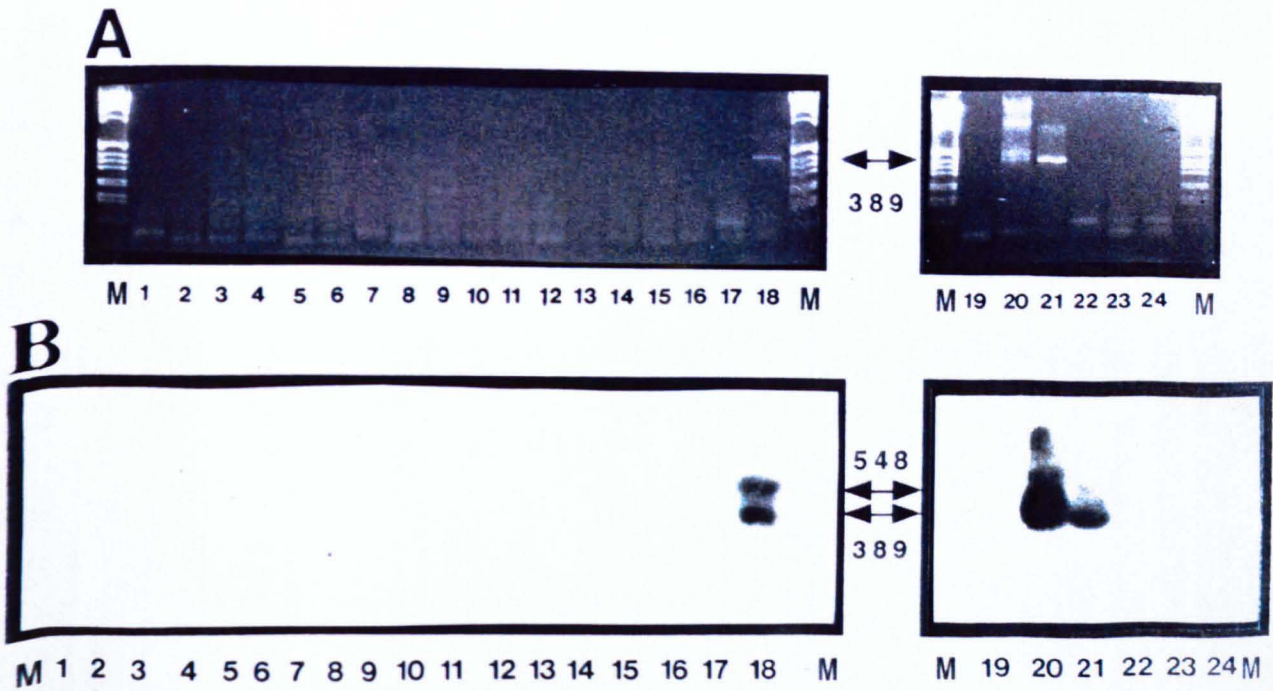


Figure 4.11. Late Gene RT-PCR of Primary Monocytes and Monocyte-Derived Macrophages (MDM). The top panel (A) shows the ethidium bromide gel of the late gene PCR. Specific sizes of the predicted products are marked with an arrow with the primary product = 548 bp and internal amplification product = 389 bp. Lanes 1 & 2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, 11 & 12, 13 & 14, 15 & 16 are paired RT-PCR samples from 4 healthy seropositive carriers and paired RT-PCR samples of MDM from 4 healthy seropositive subjects, respectively. Paired MDM samples in lanes 9 & 10, 11 & 12, 13 & 14, 15 & 16 were differentiated with PMA + HC (subject 1), PMA + HC (subject 2), GM-CSF + HC, and Vitamin D3 + PMA, respectively. Lanes 1, 3, 5, 7, 9, 11, 13, & 15 contain complete RT-PCR mixes and lanes 2, 4, 6, 8, 10, 12, 14 & 16 are RT sample controls, respectively. Lanes M are the 1 kb DNA marker. Lanes 17, 18, and 19 are RT controls as follows: RNA from 100 HCMV infected cells after RNase-free DNase treatment where lane 17 is the RT sample control, 18 is the complete RT mix and lane 19 is the complete RT mix after reverse transcription of RNA from 1 HCMV infected cell after RNase-free DNase treatment. Lane 23 is the RT H₂O control. Lanes 20, 21 and 22 are PCR controls where lanes 20 and 21 represent amplification of 1000 and 100 copies of the standard HCMV infected cell DNA stock and lane 22 is a negative H₂O control. On the Southern blot (B) probe-positive bands are seen in lanes 18, 20 and 21 which are the RT and PCR positive controls, respectively.

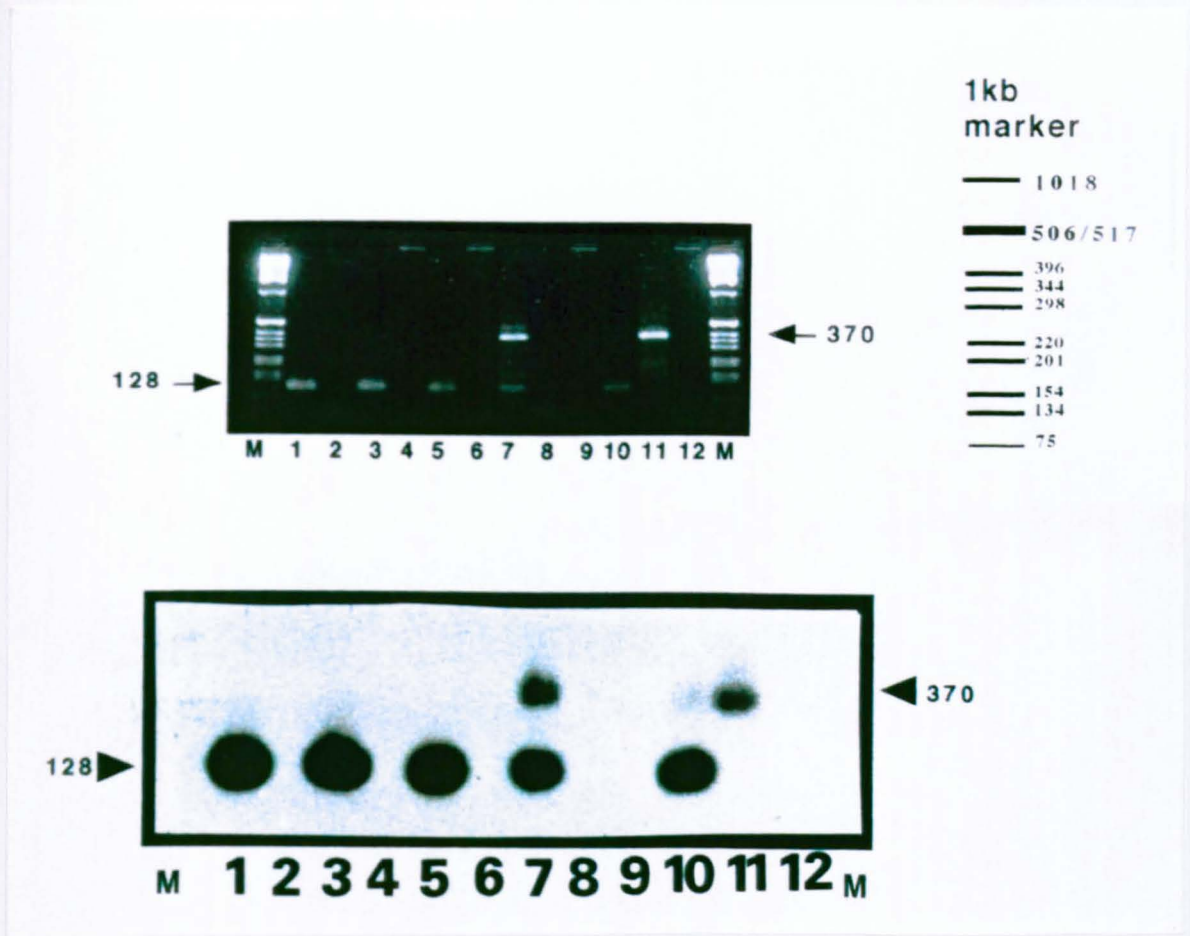


Figure 4.12. Histidyl-tRNA Synthetase Gene RT-PCR of Primary Monocytes. The figure shows the ethidium bromide gel of the Histidyl t-RNA Synthetase Gene RT-PCR at the top left and a detailed description of the 1 kb DNA marker (lanes M) at the top right. Specific sizes of the predicted products are marked with an arrow and represent DNA = 370 bp and cDNA = 128 bp. Lanes 1 & 2, 3 & 4, and 5 & 6, are paired RT-PCR samples of primary monocytes from the three healthy subjects analyzed in the other four HCMV RT-PCRs presented above (Figures 4.8, 4.9, 4.10, and 4.11) where lanes, 1, 3, and 5 contain complete RT-PCR mixes and lanes 2, 4, and 6 are RT sample controls, respectively. The 128 bp cDNA bands can be clearly seen in lanes 1, 3, and 5 while non-primed samples are negative (lanes 2,4,6). Other lanes are as follows: lanes 7 & 8 (RT-PCR of 100, and 1 HCMV infected cells, respectively), lane 9 (a H₂O RT control) and lanes 10, 11 & 12 (PCR controls containing an RT-PCR of RNA from the equivalent of 10 adherent monocytes of a healthy subject, 10 ng of DNA from a healthy subject and a PCR H₂O control).

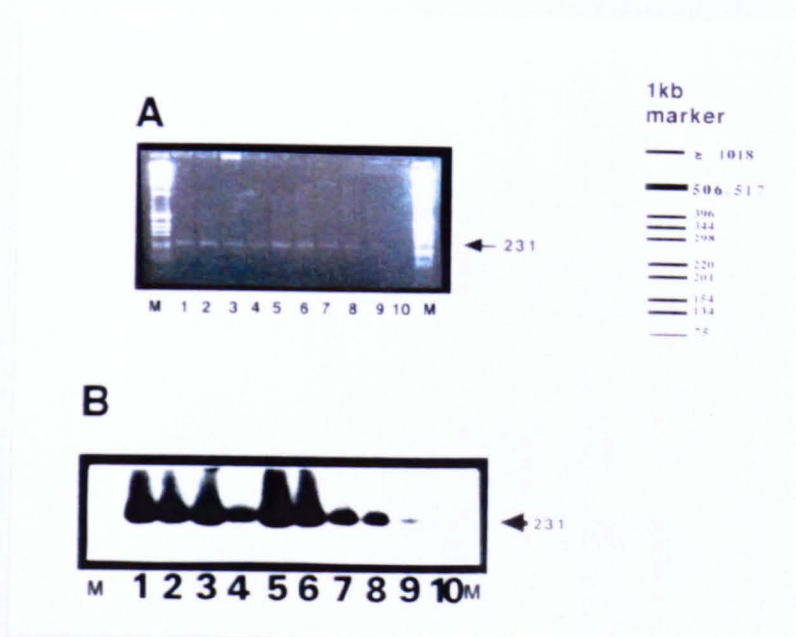


Figure 4.13. DNA PCR Control of RT-PCR. The intron-spanning IE1 PCR was used to examine DNA produced from a small aliquot of cells which were otherwise harvested for RNA for RT-PCR. The top panel (A) shows the ethidium bromide gel with the DNA product of 231 bp delineated by the arrow. The 1 kb marker (lanes M) is detailed at the right. Lanes 1 - 4 represent amplification of the 4 healthy seropositive carriers whose primary monocytes were examined for evidence of transcription (Figures 4.8, 4.9, 4.10, 4.11 and 5.5). Lanes 4-8 are the amplifications of the 4 healthy seropositive carriers whose primary monocytes were differentiated *in vitro* and examined for evidence of reactivation by RT-PCR (Figures 4.11, 5.5, 5.6, 5.7 & 5.8). In the latter instance DNA was extracted from cells after differentiation at the same time the remaining cells were harvested for RNA. Specifically, lanes 5 & 6 are PMA + HC mediated differentiation, lane 7 is GM-CSF + HC mediated differentiation and lane 8 is PMA and Vitamin D3 mediated differentiation. Lane 9 is a cDNA control (1 copy) and lane 10 is a H₂O only negative control. The bottom panel (B) shows the Southern blot of the gel in A. All bands in A are seen to be probe positive while the PCR H₂O control is probe negative

4.5 DISCUSSION

As my work had shown that monocytes were a major site of HCMV persistence in peripheral blood of healthy carriers (chapter 3) an analysis of the spectrum of HCMV gene expression in these cells was undertaken. Primary monocytes of healthy carriers showed no evidence of HCMV IE1, IE2, HQLF-1 or late polyadenylated mRNA at the level of detection used here. Although, it should be pointed out, only 4 of five possible HQLF-1 transcripts would have been detected with the PCR primer pair used here and only 3 of these would have been seen on Southern blot using a centrally located oligonucleotide probe.

Consequently whilst it cannot be ruled out that IE transcripts are present in PBMC below the level of detection used in this study, the sensitivity of the RT-PCR would suggest that PBMC of healthy carriers do not express IE RNA and rules out low level productive infection in PBMC as a mechanism of persistence of HCMV. Either, HCMV persists without any gene expression or some as yet undefined latent HCMV transcripts are expressed in these cells. Similarly the detection system used here would not have detected any non-polyadenylated transcripts.

Others have reported evidence of IE1 HCMV gene expression in healthy carriers (Schrier *et al.*, 1985) but this does not seem to be reproducible (Gnann *et al.*, 1988). And one other report (Toorkey and Carrigan, 1990) has suggested that IE1 proteins can be detected in tissues from autopsy examinations but many factors may be involved in post mortem changes triggering viral reactivation and make these results difficult to apply to persistence in healthy carriers.

In any event, the results presented here show no evidence of immediate early gene transcripts or the late gene (pp28) RNA products in monocytes from healthy carriers and, thus, no evidence of low level replicative persistence in these same cells.

CHAPTER 5

DIFFERENTIATION OF THE MONOCYTIC CELL LINE THP-1 AND ADHERENT MONOCYTES FROM HEALTHY CARRIERS TO A PERMISSIVE PHENOTYPE FOR *IN VITRO* INFECTION AND REACTIVATION OF ENDOGENOUS HCMV

5.1 INTRODUCTION

In chapter 4, it was not possible to detect evidence of transcription from endogenous virus of healthy carriers after RT-PCR of primary monocytes and as there is no *in vivo* or *in vitro* model for HCMV persistence, I chose to further investigate HCMV persistence by using a monocytic cell line (THP-1) known to be nonpermissive for HCMV in the undifferentiated state and fully permissive after differentiation (Weinshenker *et al.*, 1988). Although Weinshenker *et al.* 1988, did not clearly show that in the undifferentiated state THP-1 cells permit viral penetration, it was inferred that HCMV was able to enter the cells but was negatively regulated by cellular factors as is seen with teratocarcinoma (T2) cells (Gonczol *et al.*, 1984). Indeed, upon transient transfection, the HCMV MIE enhancer/promoter has been shown to be negatively regulated in both THP-1 cells and T2 cells *in vitro* (Shelbourn *et al.*, 1989a, Shelbourn *et al.*, 1989b, Kothari *et al.*, 1991; Sinclair *et al.*, 1992) and this repression is released upon differentiation. Moreover, there is increasing evidence that differentiation is necessary for infection of monocytes with HCMV, as *in vitro* differentiated monocyte-derived macrophages have been shown to have increased HCMV infectability (Lathey & Spector, 1991; Ibanez *et al.*, 1991). Similarly, *in vivo* terminally differentiated alveolar macrophages are 100% infectable (Drew *et al.*, 1979) compared to primary monocytes which are very difficult to infect (Rice *et al.*, 1984; Einhorn & Öst, 1984). As

it is difficult to obtain in vivo differentiated macrophages and various in vitro differentiation techniques of PBMC yield large differences in permissiveness for HCMV infection (Lathey & Spector, 1991; Ibanez *et al.*, 1991), I chose to use the THP-1 model system initially to test several in vitro monocyte differentiation techniques. Optimum differentiation conditions which permitted HCMV infection of THP-1 cells would then be applied to primary monocytes to attempt to reactivate endogenous virus. RT-PCR would be used to detect any evidence of reactivation after differentiation. Cocultivation of differentiated monocyte-derived macrophages would also be carried out to detect evidence of a full productive infection.

Molecular mechanisms involved with macrophage differentiation processes are not completely defined. In vitro it appears that monocyte differentiation is dependent on at least 2 factors, the presence of human serum and adherence (Dougherty & McBride, 1989). In the former case, 1,25 dihydroxy Vitamin D3 has been proposed as the active component in the serum (Dougherty & McBride, 1989). GM-CSF, however, is also known to play an integral role in regulation of monocytopoiesis at all levels as well as the final development of macrophages (Auger & Ross, 1992). Furthermore, treatment of monocytic cell lines with phorbol esters has been shown to induce a differentiated state that is specifically associated with increased permissiveness for HCMV infection (Weinshenker *et al.*, 1988). Finally, increased infectability of semi-permissive MDM by HCMV has been shown to be associated with hydrocortisone treatment in vitro (Lathey & Spector, 1991) and increased incidence of HCMV disease has been associated with steroid therapy in renal transplant patients (Velasco *et al.*, 1984). I, therefore, decided to analyze the differentiation of THP-1 cells with PMA, 1,25 dihydroxy Vitamin D3, GM-CSF and HC in order to maximize their permissiveness for HCMV infection, and then apply the optimal combination derived from this study of the monocytic cell line to

primary monocytes on the basis that similar treatment may permit reactivation of endogenous virus.

5.2 Materials and Methods

General materials and methods are discussed in Chapter 2. Experiments presented in this chapter were repeated at least 2 times for each method of differentiation to confirm reproducibility.

5.2.1 PMA and Hydrocortisone Treatment of THP-1 Cells

THP-1 cells were differentiated with phorbol 12-myristate 13-acetate (Sigma) and treated either with or without preservative-free hydrocortisone sodium succinate (Organon, U.K.). In addition, hydrocortisone treatment alone was used.

Hydrocortisone (HC) treatment was routinely carried out in the presence of 5×10^{-5} M HC (Pasquale *et al.*, 1989; Lathey *et al.*, 1991) in RPMI 1640 medium with 10% FCS for 7 days. Phorbol 12-myristate 13-acetate (PMA) was solubilized in DMSO at a concentration of 1 mg/ml then diluted to 10 μ g/ml in PBS and stored at -20 °C until use. PMA differentiation was routinely carried out in the presence of 2×10^{-8} M PMA (Tsuchiya *et al.*, 1982; Sinclair, *et al.*, 1992) for 12 hours. Differentiation using a combination of both PMA + HC was carried out as follows: HC was added to the cell medium at time 0, then on day 6, PMA was added to the medium for approximately 12 hours.

5.2.2 1,25 Dihydroxy Vitamin D3 Differentiation of THP-1 Cells

THP-1 cells were differentiated in the presence of 5×10^{-8} M 1,25 Dihydroxy Vitamin D3 (Biomol Research Laboratories, Inc.) over a period of 4 days in RPMI 10 medium (Koeffler *et al.*, 1989; Perkins *et al.*, 1991).

5.2.3 1,25 Dihydroxy Vitamin D3 and PMA Differentiation of THP-1 Cells

THP-1 cells were differentiated in the presence of 5×10^{-8} M 1,25 Dihydroxy Vitamin D3 (Vitamin D3) over a period of 4 days in RPMI 10 medium. On day 3, 2×10^{-8} M PMA was added overnight (approximately 12 hours).

5.2.4 GM-CSF and HC Treatment of THP-1 Cells

THP-1 cells were differentiated in the presence of 5 ng/ml (Hart *et al.*, 1991; and Dr. Jane Minton personal communication) of recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) (Genzyme) and treated with 5×10^{-5} M HC in RPMI medium over a period of 7 days.

5.2.5 Infection of THP-1 Cells

Differentiated or undifferentiated THP-1 cells were infected with HCMV strain AD169 (MOI of 10) or mock infected with medium overnight using 8-chambered tissue culture slides (Nunc), then fixed at -20°C for 3 minutes in acetone/methanol (1:1) and allowed to air dry. Although undifferentiated THP-1 cells are nonadherent, the medium could be gently aspirated without disturbing the cells and acetone/methanol fix applied.

5.2.6 PMA and HC Differentiation of Primary Monocytes

After collection, isolation, and adherence, primary human monocytes were differentiated in the presence of 5×10^{-5} M HC over a period of 6 days in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, BRL) containing 15% donor horse serum (Sera-lab), 15% FCS (Advanced Protein Products, Ltd.), 2 mM L-glutamine (Flow), 100 IU/ml Penicillin/100 $\mu\text{g/ml}$ Streptomycin (Gibco, BRL) and 3 g/L sodium bicarbonate (Flow). On day 6, 2×10^{-8} M PMA was added overnight.

5.2.7 GM-CSF and HC Differentiation of Primary Monocytes

Primary human monocytes were differentiated in the presence of 5 ng/ml of GM-CSF and 5×10^{-5} M HC for a period of 7 days in IMDM (detailed in section 5.2.6).

5.2.8 1, 25 Dihydroxy Vitamin D3 and PMA Differentiation of Primary Monocytes

Primary human monocytes were differentiated in the presence of 5×10^{-8} M Vitamin D3 (Kreutz & Andreeson, 1990) in IMDM (detailed in section 5.2.6) for a period of 7 days. On day 6, 2×10^{-8} M PMA was added overnight.

5.2.9 Infection of Primary Monocytes and MDM

HCMV M strain was used to infect MDM that had been isolated from 5 ml of blood. MDM cells were adhered to Nunc 8-chambered tissue culture slides and differentiated as described above. On the day prior to infection of the MDM cells, undifferentiated adherent monocytes were prepared on 8-chamber culture slides and incubated overnight in IMDM containing only horse serum, FCS, penicillin/streptomycin, L-glutamine, and bicarbonate. These served as undifferentiated monocyte controls. Differentiated or undifferentiated cells were infected or mock infected (with media only) overnight, then fixed at -20°C for 3 minutes in acetone/methanol (1:1) and allowed to air dry. Indirect immunofluorescence was performed as detailed in section 5.2.10.

5.2.10 Indirect Immunofluorescence of THP-1 Cells and MDM

A 1/500 dilution of an anti-IE1 murine Mab, 1D-66 (gift of Dr. Brian Rogers, Wellcome, Beckenham, UK), was applied to the fixed cells and incubated at room temperature for 1 hour. Following 2 washes in

PBS/0.1% (v/v) Tween 20, rabbit anti-mouse fluorescein isothiocyanate-conjugated F(ab')₂ fragment (Dakopatts) was applied to the cells for a further 60 minutes at room temperature. Following 2 more washes, in PBS-Tween 20, slides were air dried and Citifluor was applied prior to UV microscopy and photographing.

5.2.11 Cocultivation of MDM with Fibroblasts

One T-150 flask of MDM (differentiated as described above) was placed on ice for 10 minutes prior to gently pelleting cells into the medium. Cells were then pelleted at low speed (200 g for 5 minutes) and medium was discarded. Cells were resuspended in MEM without serum and placed onto 1 T-75 flask of 75-80% confluent (passage 26-40) MRC5 cells. A control flask of MRC5 cells of equal confluency and passage number was set up in parallel with MEM without serum. After 24 hours, medium on both flasks was changed to MEM 10% FCS. Monolayers were observed every 5 days and fed an additional 5 ml of MEM 10% FCS/T75. If there was no evidence of CPE, monolayers were passaged 1:2 after 14 days and observed for a second 14 day period. Cells were refed 10 ml of MEM 10% FCS/T150 every 5 days.

5.2.12 RT-PCR Analysis of MDM

First strand reverse transcription is detailed in section 4.2.1. IE1 PCR (section 4.2.2), IE2 PCR (section 4.2.3), HCMV late PCR (section 4.2.5) and the histidyl-tRNA synthetase PCR (section 2.11.5) have been previously detailed. The delayed-early gene, glycoprotein B (Rasmussen *et al.*, 1985; Kouzarides *et al.*, 1987), was used to further study RNA from MDM after RT. The glycoprotein B (gB) PCR was designed by Carolyn Tysoe and oligonucleotides for use in this study were kindly provided by her. The PCR was a non-intron spanning nested amplification of 244 bp (final

product) with a sensitivity of 1 copy (personal communication Carolyn Tysoe). An internal oligonucleotide was used for probing. The sequence of the primers and probe used in the gB PCR are as follows:

Forward primary: 5'> GGAAACGTGTCCGTCTTCGA <3'

Reverse primary: 5'> GAAACGCGCGGCAATCGG <3'

Forward nested: 5'> GTGTTCTGGCAAGGCATCAA <3'

Reverse nested: 5'> CGTTGATCCACACACCAGGC <3'

Probe: 5'> TACGCCAGCTGCAGTT <3'

The gB PCR reaction mixture is the same as reported for the IE amplification in section 4.2.2 with the following exceptions: primary amplification used 10% glycerol in the reaction mix along with 1.5 mM MgCl₂, while the nested PCR used 1.0 mM MgCl₂ and no glycerol.

For all RT-PCRs, RNA from MDM or primary monocytes were reverse transcribed and amplified along with matched controls which consisted of RNA and all other components of the RT mix except the RT-primer, oligo dT (denoted RT sample controls). The RT H₂O control was an RT-PCR negative control and consisted of the complete RT mix with H₂O instead of RNA, while RT-PCR positive controls were amplifications from a standard lot of RNA from HCMV infected cells which was the same throughout this study.

Finally, all RNA isolated from individual healthy carriers underwent RNase-free DNase treatment (see 2.8.2), however, in most cases for intron-spanning PCR HCMV infected cell RNA for positive RT-PCR controls was not treated with DNase. When non-intron spanning amplifications were used e.g. glycoprotein B PCR or late PCR, positive control RNA from HCMV infected cells was treated with RNase-free DNase as detailed in 2.8.2.

5.3 RESULTS: LEVELS OF PERMISSIVENESS IN THP-1 CELLS AFTER DIFFERENTIATION

5.3.1 Phenotypic Changes

After differentiation, the one consistent phenotypic change seen with all methods of differentiation was the ability to adhere to plastic whereas the undifferentiated cells remained in suspension. The only other observable change was the appearance of elongated spindle-shaped cells in the presence of 1,25 dihydroxy Vitamin D3 (Figure 5.1 & 5.2).

5.3.2 Infection in Undifferentiated and Differentiated THP-1 Cells

The level of infectability in undifferentiated and differentiated THP-1 cells was determined by using IE1 immunofluorescence (Table 5.1). The combination of cytokines leading to the greatest degree of IE1 fluorescence after differentiation and infection of THP-1 cells was PMA + HC. After differentiation, approximately 75% of the cells showed specific IE1 nuclear fluorescence, while the undifferentiated infected cells showed only rare nuclear fluorescence and uninfected differentiated cells showed no nuclear fluorescence (Table 5.1 and Figure 5.1 & 5.2). The use of HC or PMA alone showed specific nuclear fluorescence in about 10% and 20% of the cells, respectively. Second to the combination of PMA and HC, the next highest levels of infectability were seen with the combination of PMA + Vitamin D3 and GM-CSF + HC. Both these combinations showed specific nuclear fluorescence after infection in approximately 30% of differentiated cells and no nuclear fluorescence in uninfected

Table 5.1: ESTIMATED LEVELS OF PERMISSIVENESS AFTER HCMV STRAIN AD169 INFECTION OF UNDIFFERENTIATED OR DIFFERENTIATED THP-1 CELLS DETECTED BY IE1 IMMUNOFLUORESCENCE

HELFL (Fully Permissive)	100%*
THP-1 Cells (Undifferentiated)	< 1%
1,25 dihydroxy Vitamin D3	5 %
Hydrocortisone (HC)	10 %
Phorbol 12-myristate 13-acetate (PMA)	20-30 %
HC + Granulocyte Macrophage- Colony Stimulating Factor (GM-CSF)	30-40 %
PMA + Vitamin D3	30-40 %
PMA + Hydrocortisone	75-90 %

*Percentage of cells showing specific IE1 nuclear immunofluorescence after infection overnight with HCMV strain AD169 MOI of 10. These are the results of at least 2 experiments per method of differentiation.

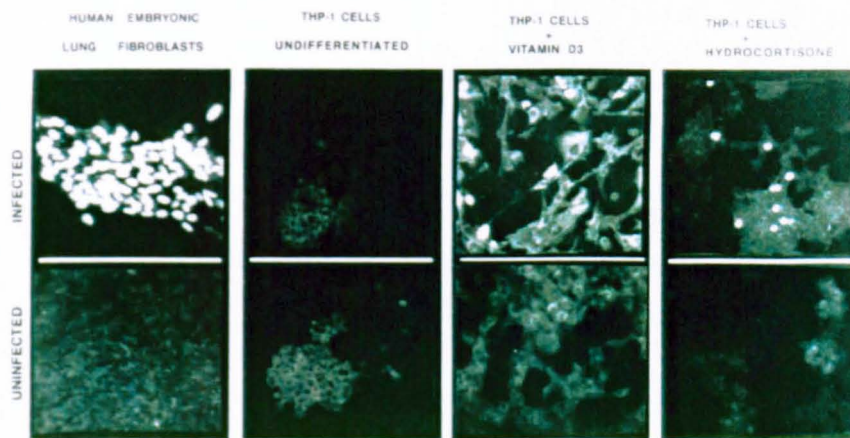


Figure 5.1. IE1 Immunofluorescence of THP-1 Cells After HCMV Infection. The panels show the results of IE1 immunofluorescence after overnight infection with HCMV strain AD169 MOI of 10. All panels are 400-fold magnification. Nuclear fluorescence can be seen in 100% of permissive HELF cells, 0% of undifferentiated THP-1 cells, approximately 5% of Vitamin D3 differentiated THP-1 cells, and approximately 10% of HC differentiated THP-1 cells (labeled at the top of the panels) after infection. No specific nuclear fluorescence is seen in any of the uninfected cells (bottom panels). The spindle-shaped phenotypic changes that occurred with Vitamin D3 differentiation can be seen.

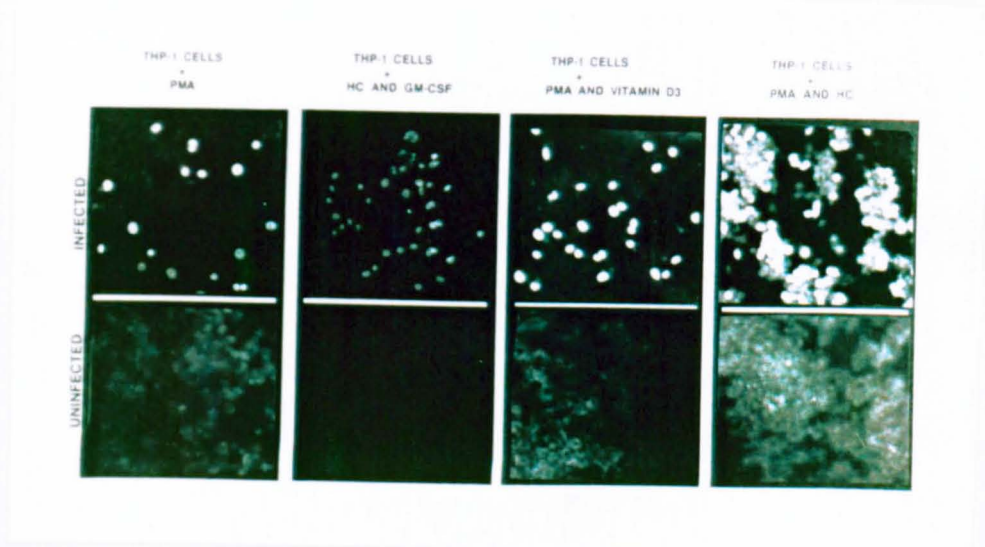


Figure 5.2. IE1 Immunofluorescence of THP-1 Cells after HCMV Infection. In these panels the results of IE1 immunofluorescence after overnight infection with HCMV strain AD169, MOI of 10 can be seen. All panels are 400-fold magnification. Specific nuclear fluorescence can be seen in approximately 20% of PMA differentiated THP-1 cells, 30% of HC + GM-CSF differentiated THP-1 cells, 30% of PMA + Vitamin D3 differentiated THP-1 cells, and 75% of PMA + HC differentiated THP-1 cells after infection. Nuclear fluorescence was not seen in any of the uninfected differentiated cells (bottom panels).

differentiated cells. When Vitamin D3 was used alone it yielded the lowest level of nuclear fluorescence after infection of only 5%.

5.4 RESULTS: *IN VITRO* DIFFERENTIATED MONOCYTE-DERIVED MACROPHAGES OF HEALTHY CARRIERS

5.4.1 Phenotypic Changes

All MDM enlarged significantly compared to primary adherent monocytes (Figure 5.3). With the combination of PMA and HC, cells showed the most varied phenotypes. Cells were spindle-shaped, rounded or irregularly shaped. Some showed large blunt pseudopods and some of the rounded cells showed multiple needle-like projects. Most cells had one nucleus but rare cells were binuclear, while no multinucleated giant cells were observed. With GM-CSF and HC most cells were rounded with many exhibiting needle-like pseudopods similar to those seen with PMA + HC differentiation and only single nuclei were observed. PMA + Vitamin D3 differentiated MDM showed marked elongation similar to that seen in the THP-1 cells (Figure 5.2) and mainly single but also some binuclear cells were observed. In addition, in the first two instances (PMA + HC or GM-CSF + HC), the cells were actively phagocytic, engulfing most platelets that had attached to the plastic flask after the initial adherence, while the PMA and Vitamin D differentiated MDM appeared to phagocytize very few of the adherent platelets (Figure 5.3).

5.4.2 Infection of Differentiated and Undifferentiated Monocyte-Derived Macrophages

A similar pattern to that seen in the THP-1 cells (Table 5.1) was observed after infection of MDM (Table 5.2), except the overall levels of IE1 immunofluorescence were lower than that observed in THP-1 cells. The

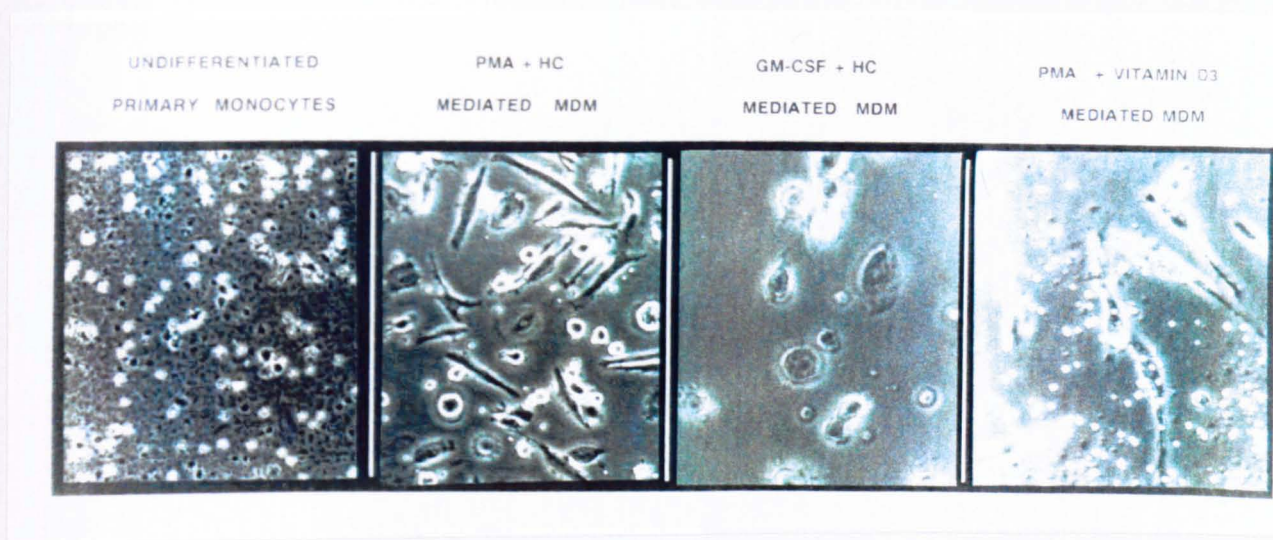


Figure 5.3. Phenotypic Changes Seen in MDM. The 4 panels show, from left to right, undifferentiated primary monocytes, phorbol 12-myristate 13-acetate (PMA) + hydrocortisone (HC) mediated differentiation, granulocyte-macrophage-colony stimulating factor (GM-CSF) + HC mediated differentiation and PMA + 1, 25 dihydroxy Vitamin D3 (Vitamin D3) mediated differentiation. All photographs represent 400 x magnification.

combination leading to the highest level of IE1 immunofluorescence after infection with HCMV M strain was PMA + HC, with an average of about 30% of the cells positive after overnight infection. Both GM-CSF + HC and PMA + Vitamin D3 showed 5-10% IE1 nuclear immunofluorescence after overnight infection. Fibroblasts appeared to be permissive in approximately 90% of cells after HCMV M strain infection, slightly less than the AD 169 strain, and the nuclear fluorescence was not as intense as that seen with AD 169 infection of fibroblasts (compare figures 5.4 and 5.1). Only rare positive cells were seen when primary monocytes were infected with HCMV M strain and none of the uninfected cell controls showed nuclear fluorescence.

5.5 RESULTS: TRANSCRIPTIONAL ANALYSIS OF ENDOGENOUS HCMV EXPRESSION IN MONOCYTE-DERIVED MACROPHAGES

5.5.1 Endogenous IE1 Transcripts are Present after PMA and HC Differentiation

Primary monocytes from two healthy seropositive individuals were examined for evidence of HCMV gene transcription after differentiation with PMA + HC. In both healthy seropositive carriers' MDM, IE1 transcripts were observed (Figure 5.5) while the respective RT-PCR sample controls were negative. From one of these subjects, 200 ml of blood was obtained at a single time point and divided in half for analysis of both primary monocytes and MDM (Figure 5.5): primary monocytes lane 9 and the RT sample control in lane 10 while RT-PCR of MDM from the same subject is shown in lane 1 and the RT sample control in lane 2. Whereas, RT-PCR from primary monocytes, lanes 11 & 12, represents a sample acquired at the same time but from a different subject than MDM RT-PCR

Table 5.2: ESTIMATED LEVELS OF PERMISSIVENESS AFTER HCMV M STRAIN INFECTION OF UNDIFFERENTIATED OR DIFFERENTIATED MONOCYTE-DERIVED MACROPHAGES DETECTED BY IE1 IMMUNOFLUORESCENCE

HELFL (Fully Permissive)	90%*
Primary Monocytes	< 1%
Hydrocortisone + Granulocyte Macrophage- Colony Stimulating Factor	10-15 %
Phorbol 12-myristate 13-acetate + 1,25 dihydroxy Vitamin D3	10-15%
Phorbol 12-myristate 13-acetate + Hydrocortisone	30-40%

*Percentage of cells showing specific IE1 nuclear immunofluorescence after infection overnight with HCMV strain M, MOI of 10. These are the results of at least 2 experiments.

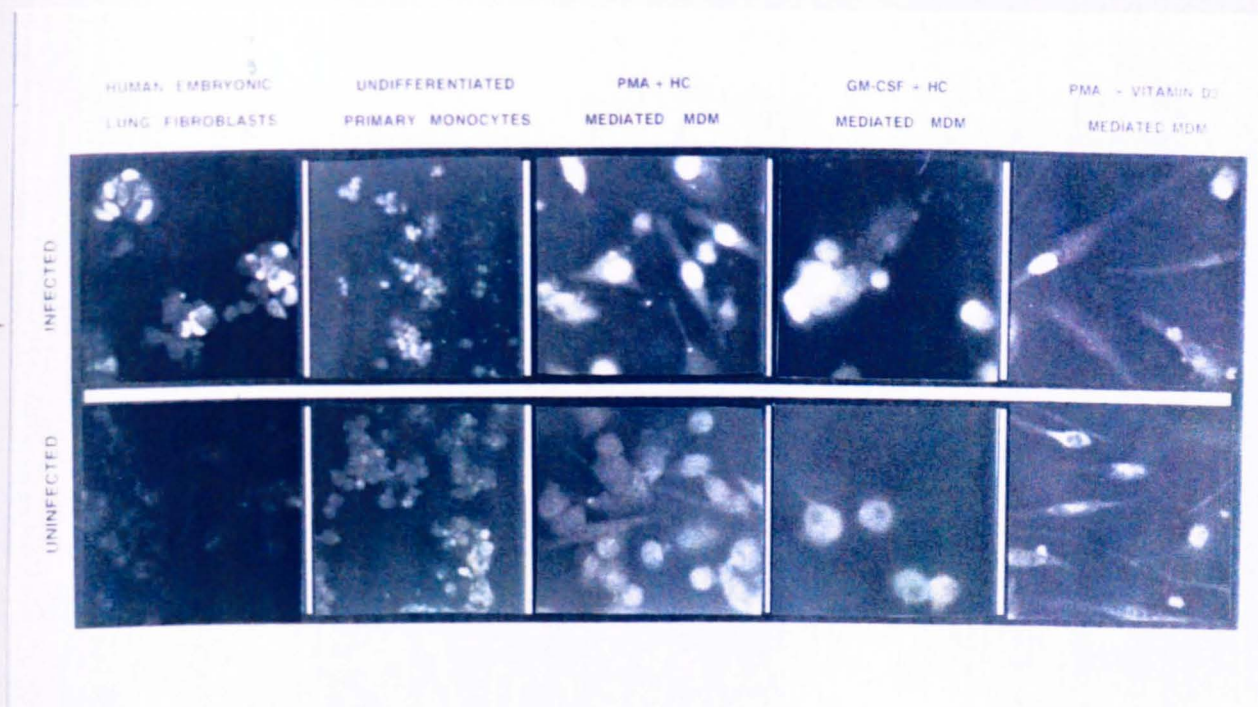


Figure 5.4. IE1 Immunofluorescence After HCMV M Strain Infection of MDM. The 5 paired panels show the results of IE1 immunofluorescence after overnight infection with HCMV strain M, MOI of 10. All panels are 400-fold magnification. The specific cells seen in each pair of panels is labeled across the top. The top row of panels are infected cells and the bottom row are the uninfected controls, respectively.

shown in lanes 3 & 4. There was no evidence of IE2 (Figure 5.7, lanes 9-12), glycoprotein B (Figure 5.8, lanes 8-11) or late transcripts (Figure 4.11, lanes 9-12) in the MDM differentiated by PMA + HC of either subject.

In both subjects' MDM, cDNA from a constitutively expressed gene, histidyl-tRNA synthetase, was detected by RT-PCR (Figure 5.9, lanes 5 & 7) while non-primed RT-PCR sample controls (Figure 5.9, lanes 6 & 8) were negative. In addition, analysis of DNA, from a small aliquot of the same differentiated MDM that was otherwise used for RNA extraction, showed that HCMV genome was present (Figure 4.13).

5.5.2 Endogenous IE, Delayed Early, But Not Late Gene Transcripts are Present After GM-CSF & HC Differentiation

Both IE1 and IE2 transcripts (Figure 5.6, lanes 1 & 2 and Figure 5.7, lanes 1 & 2, respectively), as well as, glycoprotein B transcripts (Figure 5.8, lanes 1 & 2) were detectable after primary monocytes from one healthy individual were differentiated with GM-CSF and HC. Late transcripts, however, were not detectable (Figure 4.11, lanes 13 and 14).

In Figure 5.6, where the IE1 amplification is detailed, the RT-PCR of GM-CSF + HC differentiated MDM, lane 1, shows a probe-specific 117 bp amplification product while the RT-PCR sample control, lane 2, is negative. The HCMV infected RNA control was not treated with DNase and, therefore, in Figure 5.6, lane 5, where RT-PCR of RNA from 100 cells is shown both a DNA (231 bp) and cDNA band (117 bp) are seen. However, when the RNA is diluted another 100-fold, lane 6, then reverse transcribed, the cDNA component becomes the only visible band.

In Figure 5.7, where the IE2 amplification is detailed, the RT-PCR of GM-CSF + HC differentiated MDM, lane 1 shows probe positive 266 and 406 bp cDNA amplification products while the RT-PCR sample control, lane 2, is negative. In the IE2 nested intron spanning PCR, it was not unusual to

see the primary cDNA amplification product (406 bp) carried over in the nested amplification (266 bp). This can also be seen in the RT-PCR of RNA from 100 HCMV infected cells seen in lane 3, while the RT-PCR of 1 infected cell (lane 4) and the RT H₂O control are negative (lane 5). The 1924 bp DNA bands can be seen in lanes 6 and 7 (10³ and 10² copies of standard HCMV infected cell DNA stock) while lane 8, the PCR H₂O control, is negative.

In Figure 5.8, the RT-PCR of the glycoprotein B gene can be seen. Lane 1 shows the probe positive amplification product from the GM-CSF + HC differentiated MDM, while lane 2 shows the RT-PCR sample control which is negative. The RT-PCR sensitivity is able to detect RNA from 100 but not 1 infected cell as seen in lanes 3,4,5. Probe positive amplification products from the RT-PCR positive control of DNase-treated RNA from 100 infected cells (lane 3) but not 1 infected (lane 5) can be seen, while the RT-PCR sample control for 100 infected cells (lane 4) is negative. The PCR sensitivity is 1 copy of the standard HCMV infected cell DNA stock (lane 6).

Figure 4.11, lanes 13 & 14, shown that for MDM differentiated by GM-CSF + HC, no late gene products were detected.

In the subjects' MDM, cDNA from a constitutively expressed gene, histidyl-tRNA synthetase, was detected by RT-PCR (Figure 5.9, lane 3) while the non-primed RT-PCR sample control (Figure 5.9, lane 4) was negative. In addition, analysis of DNA, from a small aliquot of the same differentiated MDM that was otherwise used for RNA extraction, showed that HCMV genome was present (Figure 4.13).

5.5.3 No Endogenous HCMV Transcripts are Present After Vitamin D3 and PMA Differentiation

After MDM differentiation from 1 healthy subject using the combination of Vitamin D3 and PMA there was no evidence of IE1 (Figure

5.6, lanes 3 and 4), IE2 (Figure 5.7, lanes 13 and 14), delayed early (Figure 5.8, lanes 12 and 13), or late gene expression (Figure 4.11, lanes 15 and 16). In addition, it did not appear as if DNA was lost during the differentiation process as DNA was detectable in the cells after differentiation (Figure 4.13).

In addition, the RT-PCR of the RNA extractions from these cells showed expression of a constitutively expressed gene (Figure 5.9, lanes 9 and 10), therefore, it appeared as if this method of differentiation was not able to reactivate any level of HCMV gene expression from endogenous virus.

5.5.4 Cocultivation Experiments with Differentiated Monocyte-Derived Macrophages and Fully Permissive HELF Cells

All MDM were cocultured with permissive fibroblasts as detailed in section 5.2.11. However, even after 4 weeks of culture, there were no plaques observed for any of the MDM/fibroblast cocultivations.

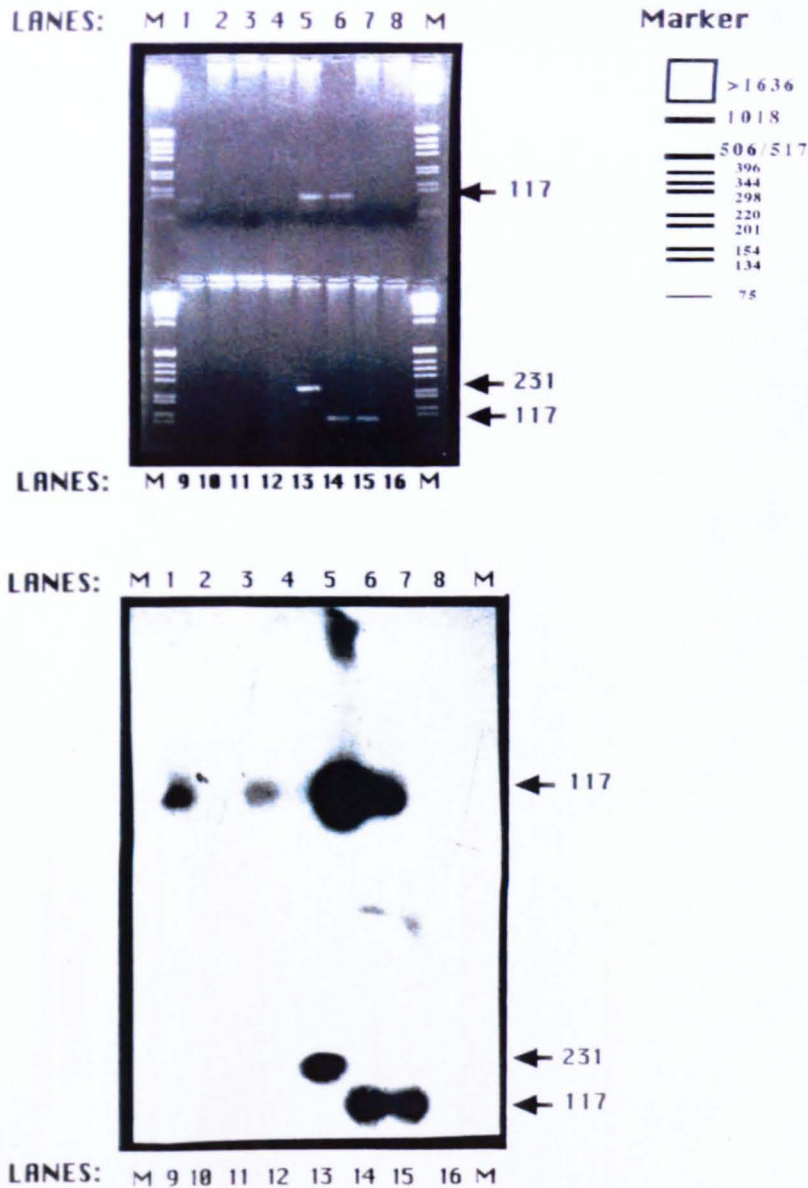


Figure 5.5. IE1 Expression of Endogenous HCMV After PMA and HC Mediated Differentiation. An ethidium-bromide agarose gel (top), the Southern blot of that gel (bottom), and a 1 kb marker (lanes M) are shown. Sizes of nested products are indicated: DNA = 231 bp and cDNA = 117 bp. Lanes 1 & 2 and 3 & 4 are paired RT-PCRs from PMA + HC differentiated MDM and lanes 9 & 10 and 11 & 12 are paired RT-PCRs from primary monocytes without *in vitro* differentiation. Lanes 1, 3, 9 & 10 are complete RT-PCRs and their respective RT-PCR sample controls are 2, 4, 10 and 12. Lanes 5, 6, 7 & 8 are RT-PCR controls as follows: RNA from 100 and 1 HCMV infected cells after RNase-free DNase treatment, and 2 RT H₂O controls, respectively. Lanes 13, 14, 15 and 16 are PCR controls as follows: 10 femtograms of the standard HCMV infected cell DNA stock, 10 and 1 copy of pGEM-2 IE1 plasmid, and a H₂O negative control.

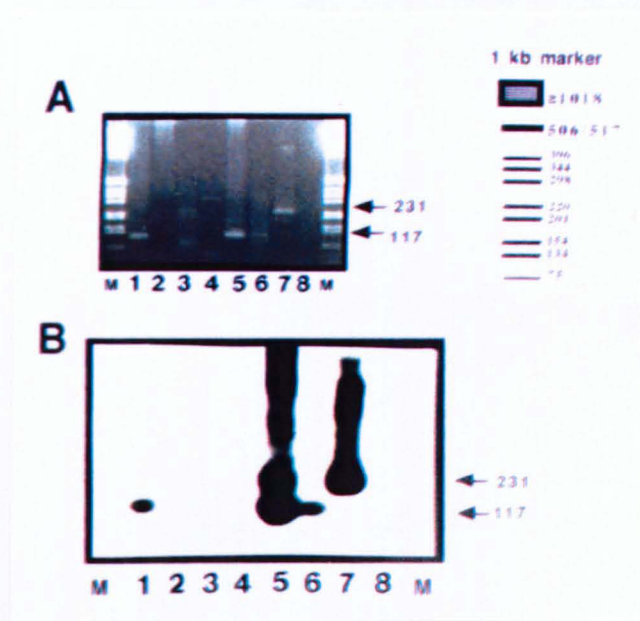


Figure 5.6. IE1 Expression of Endogenous HCMV After GM-CSF and HC Mediated Differentiation. Panel A shows the ethidium bromide gel and panel B shows the Southern blot of the gel. A 1 kb marker (Gibco) was used to estimate PCR product size (lanes M) and is detailed at the top right. Products were DNA = 231 bp and cDNA = 117 bp after nested PCR. Lanes 1 & 2 and 3 & 4 are paired RT-PCRs of MDM from healthy seropositive carriers differentiated by GM-CSF + HC and PMA + Vitamin D3, respectively. Lanes 1 and 3 are complete RT-PCRs and 2 and 4 are their respective RT-PCR sample controls. Lanes 5, 6 and 8 are RT-PCR controls as follows: RNA from 100 and 1 HCMV infected cells and an RT-PCR H₂O control. Lane 7 shows an amplification of 10³ copies of HCMV DNA.

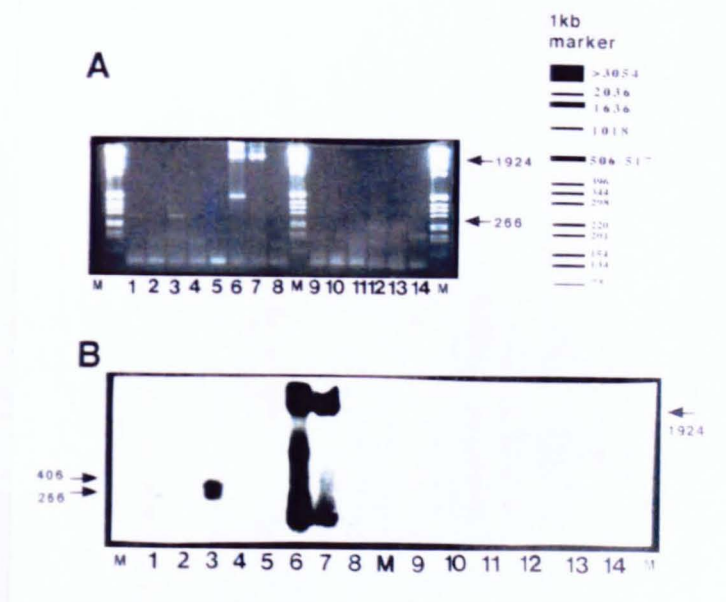


Figure 5.7. IE2 Expression of Endogenous HCMV After GM-CSF and HC Mediated Differentiation. Panel A shows the ethidium bromide gel of the IE2 amplifications and panel B shows the Southern blot of A. A 1 kb marker (Gibco) was used to estimate PCR product size (lanes M) and is detailed at the top right. Products were nested DNA = 1924 bp, primary cDNA = 406 bp and nested cDNA = 266. Lanes 1 & 2, 9 & 10, 11 & 12 and 13 & 14 are paired RT-PCRs of MDM from healthy seropositive carriers differentiated by GM-CSF + HC, PMA + HC (subject 1), PMA + HC (subject 2) and PMA + Vitamin D3, respectively. Lanes 1, 9, 11 and 13 are complete RT-PCRs and 2, 10, 12 and 14 are their respective RT-PCR sample controls. Lanes 3, 4 and 5 are RT-PCR controls as follows: RNA from 100 and 1 HCMV infected cells and an RT-PCR H₂O control. Lanes 6, 7 and 8 are PCR controls as follows: 10³, 10² and no DNA (H₂O) from the standard HCMV infected cell stock.

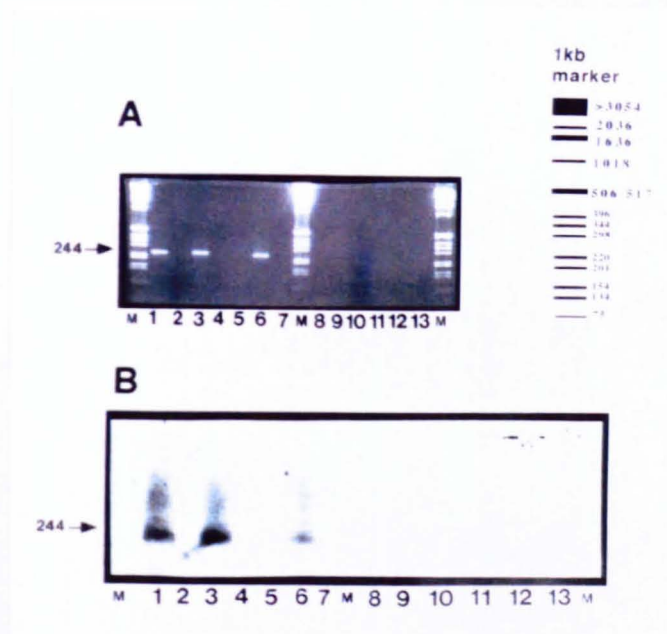


Figure 5.8. Glycoprotein B Expression of Endogenous HCMV After GM-CSF and HC Mediated Differentiation. Panel A shows the ethidium bromide gel and panel B shows the Southern blot of that gel. A 1 kb marker (Gibco) was used to estimate PCR product size (lanes M) and is detailed at the top right. Products were DNA/cDNA = 244 bp after nested PCR. Lanes 1 & 2, 8 & 9, 10 & 11 and 12 & 13 are paired RT-PCRs of MDM from healthy seropositive carriers differentiated by GM-CSF + HC, PMA + HC (subject 1), PMA + HC (subject 2) and PMA + Vitamin D3, respectively. Lanes 1, 8, 10 and 12 are complete RT-PCRs and 2, 9, 11 and 13 are their respective RT-PCR sample controls. Lanes 3, 4 and 5 are RT-PCR controls as follows: RT-PCR of DNase-treated RNA from 100 HCMV infected cells, the respective RT-PCR sample control and RT-PCR of DNase-treated RNA from 1 HCMV infected cell, respectively. Lane 6 shows an amplification of 1 copy of the standard HCMV infected cell DNA stock and lane 7 is a PCR H₂O negative control.

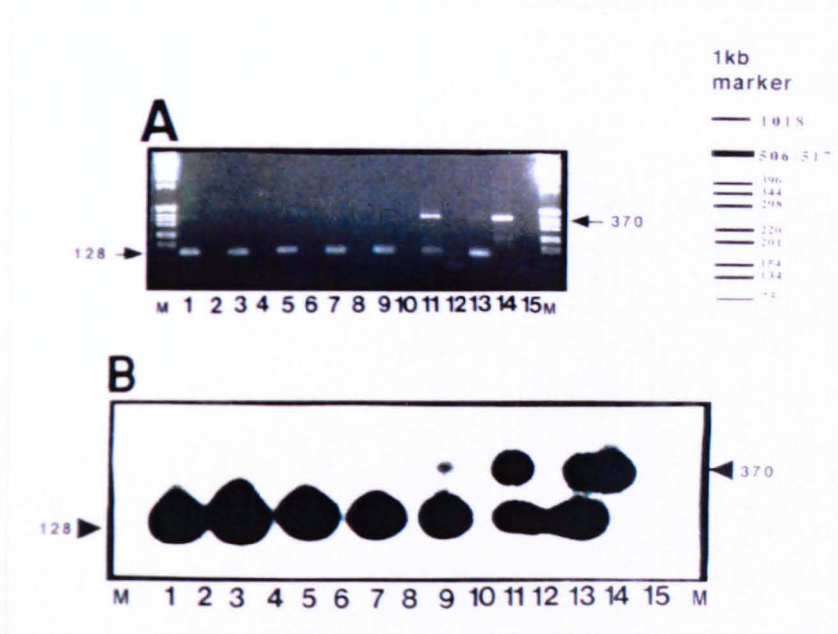


Figure 5.9. RT-PCR of Primary Monocytes and MDM using a Histidyl-tRNA Synthetase Control PCR. Panel A shows the ethidium bromide gel after histidyl-tRNA synthetase RT-PCR and panel B shows the Southern blot of the gel. A 1 kb marker (Gibco) was used to estimate PCR product size (lanes M) and is detailed at the top right. Products were DNA = 370 bp and cDNA = 128 bp. Lanes 1 & 2, 3 & 4, 5 & 6, 7 & 8 and 9 & 10 are the following: paired RT-PCR of primary monocytes from the subject shown in lanes 11 and 12 in Figure 5.5, MDM from a healthy seropositive carrier differentiated by GM-CSF + HC, MDM from a healthy seropositive carrier differentiated by PMA + HC (subject 1), MDM from a healthy seropositive carrier differentiated by PMA + HC (subject 2) and MDM from a healthy seropositive carrier differentiated by PMA + Vitamin D3, respectively. Lanes 1, 3, 5, 7 and 9 are complete RT-PCRs and 2, 4, 6, 8 and 10 are their respective RT-PCR sample controls. Lanes 11 and 12 are RT-PCR of 100 and 1 HCMV infected cells respectively. Lanes 13, 14, and 15 are PCR controls as follows: cDNA control (some DNA contamination of the non-DNase treated RNA preparation can be seen), DNA (10 ng) control and a H₂O negative control.

5.6 DISCUSSION

It is known that monocytes are difficult to infect in vitro with HCMV (Rice *et al.*, 1984; Einhorn & Öst, 1984) while in vivo differentiated alveolar macrophages are fully permissive when infected in vitro (Drew *et al.*, 1979). Furthermore, in the THP-1 monocytic cell line, permissiveness of HCMV has been correlated with differentiation (Weinshenker *et al.*, 1988). Moreover, others have reported that in vitro differentiation of primary human monocytes to monocyte-derived macrophages is necessary for of HCMV in vitro (Lathey & Spector, 1991, Ibanez *et al.*, 1991). Therefore, because analysis of primary monocytes of healthy carriers showed no evidence of endogenous HCMV transcription, as detailed in chapter 4, and because monocytes themselves are not terminally differentiated, I chose to examine methods of differentiating monocytic cells and their resulting levels of permissiveness for HCMV infection to determine the optimum method to differentiate primary monocytes in order to attempt to reactivate endogenous HCMV.

THP-1 cells were used as a representative monocytic cell line, and were differentiated using several different methods. There was little phenotypic change after differentiation other than the cells became adherent. After differentiation and infection, THP-1 cells clearly showed three combinations of chemicals associated with the highest level of infection. These were PMA + HC (75% IE1 nuclear fluorescence), GM-CSF + HC (30% IE1 nuclear fluorescence), and PMA + Vitamin D3 (30% IE1 nuclear fluorescence). All other methods of differentiation of THP-1 cells were inferior to these combinations in their ability to produce cells which were permissive for HCMV as determined by IE1 immunofluorescence.

Therefore, the 3 differentiation methods which led to the greatest percentage of THP-1 cells becoming permissive for HCMV infection were

applied to the differentiation of primary monocytes. Phenotypic changes after differentiation of primary monocytes were much more distinct than with the THP-1 cells. In general, cells took on the phenotype of macrophages: they greatly enlarged, became adherent, developed pseudopods and became phagocytic. The levels of permissiveness after infection with a recent clinical isolate (HCMV M Strain) showed similar trends to that seen with infection of the THP-1 cells but the percentage of cells showing specific IE1 nuclear fluorescence was much less. The optimum differentiation conditions resulting in the highest permissiveness of cells for HCMV infection, as determined by IE1 expression, was PMA and HC (which resulted in IE1 fluorescence in 75% of THP-1 cells and 30% of MDM). Whereas, GM-CSF + HC or Vitamin D3 + PMA resulted in similar but lower levels of IE1 expression in THP-1 cells (30% IE1 expression) and in MDM (10% IE1 expression), respectively.

When these three methods of differentiation were applied to primary monocytes of healthy seropositive carriers and RNA was analyzed by RT-PCR for HCMV gene expression, differences in the spectrum of HCMV transcripts were seen. The combination leading to the most extensive levels of HCMV expression was GM-CSF + HC. After GM-CSF + HC differentiation, the one healthy carrier who was examined showed immediate early (IE1 and IE2) and delayed early (glycoprotein B) gene expression but no late (pp28) gene expression. Cocultivation of GM-CSF + HC differentiated MDM did not show evidence of full productive infection as no plaques were detected even after 28 days of culture. It should be mentioned that these experiments were carried out on a single individual and will require further analysis of other healthy seropositive carriers. In addition, it appeared that conditions which optimized differentiation of THP-1 cells for HCMV IE expression did not correlate with the ability to reactivate endogenous virus. However, it must be remembered that the

THP-1 infection studies were optimized for IE1 expression and not virus production. In hindsight, it is possible that if conditions which maximized virus production and not IE1 expression in THP-1 cells had been studied, this would have determined conditions more optimal for endogenous virus reactivation.

In addition to GM-CSF + HC, PMA + HC differentiation of MDM was also able to reactivate HCMV gene expression in healthy carriers but to a more restricted level. Only immediate early 1, mRNA was detectable in two separate subjects after PMA + HC differentiation. While, for the combination of PMA and Vitamin D3, there was no evidence of endogenous HCMV gene expression after differentiation despite the fact that DNA was present in the cells and another constitutively expressed gene was able to be detected after RT-PCR.

It is interesting to note that in both combinations leading to endogenous gene expression after differentiation, hydrocortisone was present. Some *in vitro* data suggest that HC may act directly on the virus in tissue culture of already fully permissive fibroblasts (Tanaka *et al.*, 1984; Forbes *et al.*, 1991). This has been shown by production of increased levels of infectious HCMV in steroid treated fibroblasts over that of untreated cells. While, HC treatment of MDM prior to HCMV infection *in vitro* has been shown to increase HCMV permissiveness and productive virus release after infection in a dose-dependent manner (Lathey & Spector, 1991). This suggests that HC is influencing cellular factors allowing increased virus propagation in semi-permissive cells. Therefore, HC may have a dual effect, in potentiating HCMV gene expression from endogenous virus.

As no one has been able to reactivate HCMV from healthy carriers, this is the first report of gene expression from endogenous virus after *in vitro* differentiation. However, full productive infection was not able to be

shown by late gene RT-PCR or coculture techniques. It may be that specific cellular interactions are necessary for endogenous HCMV reactivation in vivo. Or, it may be that other cytokines are important in vivo for endogenous HCMV reactivation, and the right combination of these chemicals were not used to produce a full productive infection. Finally, as the kinetics of HCMV infection in MDM are not known it may also be conceivable that cells were harvested too soon after GM-CSF + HC differentiation (7 days) to see full reactivation and late gene expression. The same might be concluded for the use of PMA + HC, particularly since the cells were harvested only 12 hours after the addition of PMA.

CHAPTER 6

CONCLUDING DISCUSSION

6.1 DISCUSSION

In this analysis, HCMV persistence in the healthy carrier was investigated. First, specific cell types which harbored HCMV in the healthy carrier were analyzed. As extensive circumstantial evidence suggested that HCMV exists in white cells of the peripheral blood, this tissue was examined in asymptomatic adult volunteers. In addition, as persistent HCMV would most likely be present in low copy number a highly sensitive technique, nested PCR, was applied. Using purified cell populations, the predominant site of HCMV persistence in the peripheral blood of seropositive and some seronegative healthy carriers was found to be the monocyte. This data is in contrast to the previously reported T cell site of persistence in PBMC of healthy subjects (Schrier *et al.*, 1985). However, from my analysis of one seropositive individual with weak positive signal in the CD3+ cells, there was evidence that a minor site of persistence might be the CD8+ T cells of the peripheral blood while CD4+ T cells showed no evidence of HCMV persistence in this subject. This is also in contrast to Schrier *et al.* 1985, who reported that the predominant HCMV signal was found in the OKT4 cells. On the other hand, the results of my analysis would be consistent with Toorkey & Carrigan's, (1989) data that HCMV is present in tissue macrophages. Approximately 30% of HCMV seronegative healthy subjects examined in my analysis showed consistent evidence of PCR detectable sequences in adherent monocytes over a period of 2 or more months, while the individuals' serostatus remained negative. In one of these individuals an identical pattern to that seen in seropositive/PCR + subjects (e.g. after FACS and PCR amplification, the HCMV probe-positive signal was much greater in the

CD3- population as opposed to the CD3+ population) was detected. Seronegative but HCMV PCR positive subjects have been reported by others and the incidence reported in this study (30%) is consistent with other reported studies where 16 to 34% of healthy adults were found to be consistently seronegative and PCR positive (Stanier *et al.*, 1989; Bevan *et al.*, 1991; Stanier *et al.*, 1992). Finally, granulocytes have not been specifically studied in healthy carriers using a highly sensitive detection technique before this analysis. Yet, it is well documented that HCMV can be detected in high numbers of PMNL of viremic immunocompromised patients with active disease and in rare instances in immunocompetent subject who are symptomatic with HCMV infection. Such immunofluorescent data suggesting that PMNL were actively infected with HCMV have, however, recently become unreliable due to the fact that many monoclonal antibodies which were believed to detect IE antigen are actually specific for a viral structural protein (Revello *et al.*, 1992; Grefte *et al.*, 1992). Although, some evidence does suggest that a small number of PMNL in viremic patients may express HCMV IE, E, or late transcripts as detected by *in situ* hybridization as well as IE, and very rarely, late antigens as detected by immunofluorescence (Revello *et al.*, 1992), it appears that most PMNL in viremic patients are not actively infected with HCMV and intracellular viral particles are primarily phagocytized (Revello *et al.*, 1992; Grefte *et al.*, 1992). In agreement with this, was the observation in this study that polymorphonuclear cells in healthy carriers who harbor persistent virus showed no evidence of HCMV genome despite that fact that adherent monocytes from the same subjects consistently showed HCMV probe-positive signals. As it is difficult to infect freshly isolated monocytes with HCMV and in the few cells that can be infected virus does not appear to replicate, the issue arises as to how these cells may acquire and maintain persistent HCMV. It is known that bone marrow

progenitors can be infected in vitro, therefore, one explanation of monocytic cell persistence may be that the cells acquire HCMV at an early stage of their differentiation in the bone marrow. However, the same myeloid precursors give rise to both monocytes and PMNL, but PMNL do not appear to harbor HCMV in healthy carriers whilst monocytes do. Therefore, if early myeloid precursors are targets for HCMV infection it would appear that differentiation results in a partitioning of HCMV to the monocyte fraction. One explanation for this could be that HCMV infected PMNL precursors may be unresponsive to colony stimulating growth factors resulting in a loss of these cells (Sing & Ruscetti, 1990).

Following analysis of the sites of persistence in the peripheral blood of healthy carriers, I then analyzed these cells for evidence of transcriptional activity. As previous data addressing this same question has been inconclusive (Schrier *et al*, 1985; Gnann *et al.*, 1988) or had analyzed tissues that were not directly reflective of the healthy state (Toorkey & Carrigan, 1989), I chose to analyze primary monocytes obtained directly from healthy carriers. In addition, I studied both immediate early and late transcripts in these cells by using the highly sensitive system of reverse transcription coupled with nested PCR. However, in no case did I find evidence of polyadenylated mRNA in primary monocytes from either the HCMV IE1 or IE2 transcription units, the HCMV HQLF-1 IE gene or the HCMV pp28 late gene in 3 healthy seropositive carriers. Despite this, all RT-PCR samples demonstrated a cDNA product from the constitutively expressed histidyl-tRNA synthetase gene. Moreover, all DNA samples obtained from the same cells which were extracted for RNA were positive for HCMV genome. Thus, in the healthy asymptomatic carrier it does not appear that HCMV low level replicative persistence occurs. However, I was not able to rule out whether non-polyadenylated mRNA or other, as

yet, undetermined latent transcripts not specifically examined existed in primary monocytes from healthy carriers.

It is known that monocytes circulate in the peripheral blood for an average of 70 hours before diapedesing across endothelial borders and migrating to peripheral organs where terminal differentiation to macrophages occurs (Whitelaw, 1966). In addition, it has been reported that in several in vitro cell systems, differentiation is needed for fully permissive infection with HCMV. In particular, the embryonal teratocarcinoma (T2 cells) cell line, that differentiates upon retinoic acid stimulation into several somatic cell types, has been shown to repress viral transcription at the IE level (Nelson & Groudine, 1985; LaFemina & Hayward, 1986) but with retinoic acid differentiation can produce fully productive infection and release of progeny virus (Gonczol *et al.*, 1984). In addition, this is not due to a block in viral penetration (Gonczol *et al.*, 1984; LaFemina & Hayward, 1986). Moreover, Kothari *et al.* (1991), have shown that two upstream regions of the MIE responsible, at least in part, for this block and which act through binding of a potential negative regulatory factor(s) are the imperfect dyad (approximately -962) and the 21 bp repeat elements present in the enhancer. A similar analysis (Sinclair *et al.*, 1991) was performed in a monocytic cell line, THP-1, where the phorbol ester (PMA) was used to differentiate the cells to macrophages. This report showed the same results as those seen in the T2 cells, in that a potential candidate negative regulatory factor(s) existed in these human monocytic cells and that deletion of the binding sites for this factor allowed expression of the MIE promoter in undifferentiated THP-1 cells. It is possible that such negative regulatory factors also exist in primary monocytes and could be the reason for the lack of expression of the MIE promoter in primary monocytes seen in my analysis

Because no HCMV gene expression could be seen in primary monocytes from healthy seropositive carriers, precluding an analysis of the mechanism of HCMV regulation during persistence in primary monocytes, I decided to analyze methods of differentiation in a monocytic cell line as well as primary monocytes in an attempt to reactivate persistent virus. Whilst, it is difficult to infect primary monocytes (Rice *et al.*, 1984; Einhorn & Öst, 1984), it is known that differentiation leads to increased permissiveness to HCMV infection in monocytic cell lines (Weinshenker *et al.*, 1988) as well as for *in vitro* and *in vivo* differentiated macrophages (Drew *et al.*, 1979; Lathey & Spector, 1991; Ibanez *et al.*, 1991). Specifically, HCMV permissiveness in undifferentiated and differentiated adherent peripheral blood mononuclear cells (Lathey & Spector, 1991; Ibanez *et al.*, 1991) have shown that hydrocortisone and T cell stimulation, using PHA in the first case, and T cell stimulation using Concanavalin A, in the second case, prior to infection can significantly increase the number of cells expressing IE antigens and the number of cells productively infected. When Concanavalin A stimulated T cells were used as a method of monocyte differentiation, it is not unreasonable to speculate that at least one cytokine mediating differentiation was GM-CSF, as this is a major by-product of T cell stimulation by Concanavalin A (Clark & Kamen, 1987). Moreover, both Lathey & Spector, (1991) and Ibanez *et al.* (1991) used human serum in the culture medium to produce MDM and it has been speculated that 1,25 dihydroxy Vitamin D3 is one factor present in human sera necessary to mediate differentiation of monocytes (Dougherty & McBride, 1989). I, therefore, decided to try combinations of the following differentiating agents in my analysis of endogenous HCMV reactivation: phorbol 12-myristate 13 acetate, granulocyte-macrophage colony stimulating factor, hydrocortisone and 1,25 dihydroxy vitamin D3.

In my analysis I chose to examine methods of in vitro differentiation resulting in a differentiated macrophage phenotype permissive for HCMV IE expression using the monocytic cell line, THP-1. In these experiments, it was found that 3 combinations of differentiation-mediating chemicals (PMA + HC, GM-CSF + HC & PMA + Vitamin D3) were able to produce the highest level of HCMV permissiveness as determined by IE1 fluorescence. Extrapolating from these results, the same three combinations of differentiation-mediating agents were used with primary monocytes from healthy seropositive donors in both infection studies and in studies attempting to reactivate endogenous HCMV virus from differentiated monocyte-derived macrophages. IE1 immunofluorescence was also used to assess results of infection studies in the MDM cells and similar but lower levels of permissiveness as that seen in the THP-1 cells were found. PMA + HC lead to the highest level of IE1 immunofluorescence (75% of THP-1 cell and 30% of MDM), while GM-CSF + HC and Vitamin D3 + PMA both lead to 30% IE1 expression in THP-1 cells and 10% IE1 expression in MDM. However, when RNA from MDM differentiated by these same three combinations of chemicals were studied by RT-PCR, it was found that GM-CSF + HC lead to the most extensive induction of HCMV endogenous gene expression as determined by RT-PCR analysis. In the single healthy individual studied, both IE1 and IE2 polyadenylated mRNA transcripts were detected as well as delayed-early HCMV gB gene transcripts. However, HCMV late gene (pp28) mRNA was not detectable after RT-PCR which was consistent with the cocultivation studies where GM-CSF + HC differentiated MDM passaged onto permissive fibroblasts lead to no evidence of plaques even after 28 days. The second most successful combination of differentiating agents leading to expression of endogenous HCMV was PMA + HC. With this combination, only IE1 expression was seen and cocultivation studies were negative. Finally, the combination of

PMA and Vitamin D3 lead to no detectable mRNA transcripts in MDM and cocultivation studies were negative. In all cases, successful RT-PCR was verified by the amplification of a constitutively expressed gene, histidyl-tRNA synthetase. As well, in all cases a nested IE1 DNA PCR was used to verify that the cells harboring HCMV had not been lost in the differentiation process.

It is not surprising that the infection analysis of THP-1 cells after differentiation did not parallel results of HCMV endogenous gene expression in MDM, because the former analysis was based on an optimization of IE1 expression. Retrospectively, optimization of differentiation of THP-1 cells for productive infection might have resulted in different conditions to optimize production of infectious virus in MDM. In any event, I report in my analysis of HCMV viral persistence in the healthy human carrier, the first observation of reactivation of endogenous HCMV, albeit to the level of delayed-early gene expression.

Finally, although in my study I have addressed the peripheral blood compartment with respect to HCMV persistence, epithelial cells are also thought to be another site of HCMV latency. In particular, ductal epithelium of the salivary glands (squamous epithelium of the buccal or pharyngeal mucosa have not been examined), renal tubular epithelium and cervical epithelium are thought to be the source of recurrent HCMV shedding from the saliva, urine and cervix, respectively, and this shedding is known to occur sporadically throughout the life of many healthy carriers (Nankervis & Kumar, 1978; Alford & Britt, 1990). Although these cells have not directly been shown to be the source of infectious HCMV shed from healthy carriers, two pieces of evidence suggest the likelihood of this. Firstly, epithelial cells of the cervix from HCMV seropositive female carriers have been found to show evidence of HCMV by in situ hybridization detection in the basal cells of the squamous

epithelium from biopsy of the cervix taken at colposcopy (Mathijs *et al.*, 1991). Unlike the findings with EBV (see Allday & Crawford, 1988 for review), however, the more differentiated superficial cells did not show positive in situ signal. Second, Toorkey & Carrigan, 1989, have suggested that tubular, bronchial and alveolar epithelial cells harbor HCMV in autopsy samples from ante mortem healthy subjects. In their analysis, salivary glands were examined in only 1 subject and did not show evidence of HCMV while cervical tissue was not examined. If epithelial cells do harbor HCMV in the healthy carrier, it will be important to determine whether this represents a true site of latency and whether interaction between HCMV-bearing macrophages plays a role in the shedding of virus from asymptomatic carriers.

6.2 FUTURE AIMS

In the analysis of sites of persistence, it will be necessary to study further subjects before determining whether T cells represent a minor site of persistence. As the bead-sort method did not allow for an easily interpretable level of purification of cell populations it may be more productive to approach this analysis by using FACS in combination with PCR as was used for the analysis of the T cell and non-T cell populations.

In addition, the analysis of persistent gene transcripts in primary monocytes would be more complete if random hexanucleotide RT primed samples were analyzed. In this case, non-polyadenylated transcripts would be able to be detected.

Finally, the analysis of reactivation of endogenous HCMV using various methods of monocyte differentiation requires further examination. Firstly, more individuals' MDM should be examined using the combination of GM-CSF + HC and PMA + HC for differentiation to both repeat previous work and extend the time in culture to allow for any slower

kinetics of infection in MDM after reactivation of endogenous virus. By using blood from seropositive healthy donors received from the transfusion service, enough cells could be obtained to analyze the same blood sample for evidence of gene transcription in primary monocytes as well as MDM and verify that the subject is not constitutively expressing HCMV genes. Ultimately, further investigation of the possibility of producing an in vitro fully productive viral reactivation of endogenous persistent HCMV will be required. This may need both specific cellular interactions as well as the use of other cytokines. Certainly, IL3 has been shown to stimulate formation of macrophages and with the exception of the WEHI-3 myelomonocytic cell line and perhaps epidermal cells, IL3 is exclusively produced by activated T cells (Luger *et al.*, 1985). In the system presented in this report, T cells would not have been present in any large number and would not have been specifically activated. Also, they would have been rendered non-functional by the use of hydrocortisone. Consequently, IL3, along with GM-CSF + HC or PMA + HC may be necessary to produce a differentiated MDM more reflective of in vivo macrophages and thus able to fully reactivate endogenous virus. Finally, by using Concanavalin A-stimulated-T-cell-conditioned medium, all cytokines produced by activated T cells should be present and may, ultimately, be the simplest approach to attempt to differentiate monocytes and reactivate endogenous HCMV.

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