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A NESTED PCR FOR THE DETECTION OF CMV IN BLOOD MONONUCLEAR CELLS FROM HIV INFECTED PATIENTS

by

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THESIS
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of the requirements for the degree of
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ABSTRACT

The Human Cytomegalovirus, a member of the Herpesvirus family, is a relatively non-pathogenic virus except in those individuals whose immune systems have not fully developed (newborns) or are immunosuppressed (transplant patients) or immunodeficient (individuals with AIDS). Its direct effects in the latter group include infections of the retina potentially causing blindness and in the gastrointestinal tract.

Given its pathogenicity in HIV seropositive and transplant patients and because of its proposed role as a viral cofactor in AIDS disease progression, the virus has and is being intensively studied. A rapid test to detect the virus in clinical samples is needed in order to assess the status of an HCMV infection and to monitor the virus in an individual over time.

Faced with the alternative detection systems such as viral culture, immunofluorescence assays and DNA probing, the polymerase chain reaction is an attractive choice because of its rapidity and unrivalled sensitivity.

Nested polymerase chain reaction for HCMV was used in the present work in order to determine how useful it is in the detection of the virus in peripheral blood samples. Two nested assays were evaluated and optimized using blood samples from HIV seronegative blood donors, HIV-1 seropositive asymptomatic individuals and individuals with AIDS. The results show that the assay is rapid, sensitive and specific. Furthermore, the virus was readily detectable by PCR in individuals with AIDS while it is infrequently found in the other two groups. Finally, the PCR results were positive 14 and 7 months, respectively, in two individuals with AIDS prior to the development of overt HCMV mediated disease

suggesting that PCR is a useful monitoring tool in individuals at high risk for HCMV disease.

PCR has many applications in basic and applied research and potentially in the detection of infectious diseases. The results here suggest that its use may help to predict who may and may not develop the potentially sight and life-threatening diseases caused by HCMV.

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
bp	base pairs
CID	Cytomegalic Inclusion Disease
CMV	cytomegalovirus
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocyte
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	Early
EBV	Epstein Barr Virus
ELISA	Enzyme Linked Immunosorbent Assay
gc	glycoprotein
GI	Gastrointestinal
HCMV	human cytomegalovirus
HHV-6	Human Herpesvirus-6
HIV	Human Immunodeficiency Virus
HSV-1	Herpes Simplex Virus type 1
HSV-2	Herpes Simplex Virus type 2
IE	Immediate Early
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IR_L	Internal Repeat of long segment

IR_s	Internal Repeat of short segment
kb	kilobase
kDa	kilodalton
L	Late
MHC	Major Histocompatibility Complex
mIE	major Immediate Early
mu	map units
µg	microgram
µ*M*	micromoles per litre
mL	millilitre
m*M*	millimoles per litre
mRNA	messenger ribonucleic acid
MOI	Multiplicity of Infection
pp	phosphoprotein
PBMC	peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PMNL	polymorphonuclear leukocyte
$TCID_{50}$	50% Tissue Culture Infectious Dose
TR	Terminal Repeat
UL	Unique long
Us	Unique short

I. INTRODUCTION

I.1 PREAMBLE

The study of the human cytomegalovirus (HCMV) is many decades old. This introduction begins with a brief review of the discovery of the virus, followed by a review of its general characteristics, epidemiological features, and pathology. Following this, a review of the revolutionary molecular biology technique—the polymerase chain reaction (PCR) — is given with a view towards its use as a tool in the detection of infectious diseases, particularly HCMV.

HCMV, while being essentially harmless in"normal" individuals can be extremely pathogenic in immunocompromised and immunologically immature individuals. A rapid method of detection of the virus is therefore essential in order to provide for and monitor anti-HCMV therapy. As we shall see, PCR may be a unique tool in which to diagnose and evaluate the state of a HCMV infection.

I.2 THE HISTORY OF HCMV

Monto Ho, in his comprehensive review of the virus (1), divides early research on the virus into two separate phases. The first period he calls the *period of cytopathology* while the second he calls the *virological period.*

The period of cytopathology began in 1905 with the observation of abnormally large cells—"protozoan- like cells" in the lungs, kidneys and liver of an 8 month old fetus

associated with lesions of hereditary syphilis. These cells contained cytoplasmic and intranuclear inclusions that were first believed to be of non-human origin. Various case reports followed which continued to find these abnormal large cells (1). The term "cytomegalia" was first introduced in 1921 by Goodpasture and Talbot (2) to describe these cells and the authors believed they were not protozoa but were large mononuclear cells. By 1925, Von Glahn and Pappenheimer (3), in a case study, made the association between the intranuclear inclusions and infection by herpesvirus and concluded that the inclusions seen in their patient must be caused by a herpesvirus or a closely related virus.

Further work demonstrated that inclusion bodies were caused by certain virus infections (4). After finding inclusion containing cells in renal tubules of congenitally and lethally infected children, Wyatt *et al* (5) suggested that these cells might be detectable in the urine of afflicted infants and in 1952, Fetterman (6) reported such a finding from a 3 day old premature infant. Cytologic examination of the urine then became the most sensitive and specific method of laboratory diagnosis. In 1953, Minder (7) used electron microscopy to examine cytomegalic inclusion cells and found 100 nm particles in the cytoplasm and in the clear halo surrounding the intranuclear inclusion. This was the first time that human CMV was seen.

The so-called *virological period* began with the ability to culture the virus *in vitro.* This development had to wait until human cells could be routinely grown in culture since HCMV will not grow in experimental animals or non-human cells. Following this tissue culture advancement by Enders *et al* in 1949 (8), three groups more or less simultaneously

and independently reported the culture of HCMV and two of them produced a well known laboratory strain that is used today. Smith in 1956 (9) was able to culture HCMV from the salivary gland and kidney of infants who had died of cytomegalic inclusion disease. Rowe *et al* (10) were studying adenoviruses by culturing adenoidal tissue. One of the cultures, however, had an unusual type of cytopathology which resembled the cytopathic effect (CPE) of Weller *et al* (11). In actual fact, after comparing the cultures, it was concluded that Rowe *et al* had cultured a HCMV strain, now known as HCMV strain AD169 while Weller's cultures had given rise to the Davis strain of HCMV.

Since this time, much more study on the epidemiology, immunology and molecular biology of the virus has been carried out. The AD169 strain, for instance, has been completely sequenced (12) and much work is focusing on the newly identified open reading frames and the potential proteins that they encode. But as will become evident, despite intensive study, many questions remain unanswered about the virus.

I.3 HCMV CLASSIFICATION

The virus family *Herpesviridae* contains nearly 100 herpesviruses that have been isolated from various animal species (13). Of these, six are known to infect humans: Herpes Simplex Virus types 1 & 2 (HSV-1, HSV-2); Varicella- Zoster (VZV), Epstein-Barr (EBV) and Human Herpes Virus 6 (HHV-6). Human Herpes virus 5 is the cytomegalovirus that infects humans. Cytomegaloviruses are well known for their extreme species specificity as HCMV will not infect other species and vice-versa. CMV strains have been found in non

human primates, birds, fish, cows, guinea pigs, mice—in almost every animal species studied to date (13).

Viruses are classified as herpesviruses based on the molecular structure of their virion. They all have a lipid envelope which surrounds a tegument and an icosahedral capsid containing a large linear, double stranded DNA genome (13).

CMV, along with the other herpesviruses share the characteristic of life long infection of the host and periods of latency and reactivation (13). Upon primary infection of the host, these viruses are not completely cleared by the body and remain "hidden" in certain tissues of the body. Certain stimuli, for example immunosuppression, can allow the virus to begin replication again until they can once again be brought under control by the immune system.

I.4 THE STRUCTURE OF THE HCMV VIRION

HCMV virions are pleiomorphic, ranging from 150 to 300 nm in diameter (1). The core consists of a linear double-stranded DNA genome of 230 000 base pairs (bp) that is surrounded by an icosahedral capsid. The capsid is composed of 162 capsomeres with the predominant protein being the **major capsid protein** of 150 kDa. Surrounding the nucleocapsid is the tegument or matrix which is a fibrous, granular array of proteins between the nucleocapsid and envelope. The predominant phosphoproteins found in the matrix include the **lower matrix protein** (65 kDa, pp65), **upper matrix protein** of 71 kDa (pp71) and a 150 kDa basic phosphoprotein (pp150) (14). There are at least nine additional phosphoproteins found in the matrix in smaller amounts, ranging in size from 24 to 122 kDa

(14). The matrix proteins probably serve to anchor the envelope to the nucleocapsid and may have roles in regulating DNA synthesis or viral assembly (1). Finally, a lipid bilayer envelope surrounds the virion and contains 3 main multimeric complexes of viral glycoproteins named gcI, gcII and gc III. Also, there are at least two other envelope proteins, one is known as the **internal membrane glycoprotein**, so-called because of its multiple membrane spanning domains (15) and a 48 kDa glycoprotein (16).

The gcI complex contains glycoproteins of 130, 93 and 50-52 kDa which are linked by disulfide bonds (17). The 52 kDa protein shows homology with the glycoprotein B of HSV and other herpesviruses and so is also known as the gB homologue (1). gcII, first described in 1986 (18), is a complex of glycoproteins with molecular weights of 47-52 kDa and is linked to two antigenically related glycoproteins of 90 and greater than 200 kDa (17). The gcIII complex migrates homogeneously in sucrose gradients as a complex of 240 kDa but actually consists of 2 subunits called gp86 and gp145 (1). gp86 is known as the gH homologue because of its homology to the gH glycoprotein of HSV.

The functions of these proteins are not well characterized but because some of these proteins have homologies to HSV proteins whose functions have been better defined, it is tempting to ascribe these functions to HCMV proteins as well. In HSV, for example, gB is involved in viral entry and cell fusion and is required for infectivity at a stage after viral attachment but before expression of viral proteins (1). Similarly, gH may be involved in either intracellular spread or initiation of virus infectivity (1). No known function has been attributed to gcII, although it has been implicated in the binding of HCMV to cells with

attributed to gcII, although it has been implicated in the binding of HCMV to cells with heparin on their cell membranes (19).

I.5 INFECTIOUS CYCLE

HCMV, when compared to other viruses, replicates very slowly. Comparing HCMV to HSV, for example, the first detectable release of virus occurs 120 hours post infection for HCMV while it takes HSV only 8 hours post infection *in vitro* (1). Adsorption and penetration seem to take the same amount of time but subsequent replicative events take much longer. It is not known why, but it maybe due to the greater complexity of the virus (1).

The infectious cycle begins as follows: the virus first adsorbs to the target cell. The specific receptor has not been identified, although some have hypothesized that it may bind to MHC class I via β_2-microglobulin because β_2-microglobulin binds to HCMV (20, 21). However, others have identified two cell membrane proteins of 32 and 34 kDa that are necessary for HCMV to bind to fibroblasts (22-24). Initially, these proteins were thought to be expressed only on fibroblasts and lymphocytic cell lines but Nowlin *et al* (24) have found a wider cell distribution including epithelial and monocytoid cell lines. Yet another group has suggested that HCMV binds to heparin via the gcII glycoprotein complex (19). Perhaps there exists more than one receptor for HCMV.

In any event, after adsorption, the envelope is thought to fuse to the cell membrane. This fusion may be mediated through the gH glycoprotein binding to its cell membrane

receptor (25, 26) because monoclonal anti-idiotype antibodies that mimic gp86 prevent HCMV fusion to cell membranes but does not prevent viral attachment to the cells. Following fusion, the de-enveloped capsid is transported to the nuclear pores, where DNA is released into the nucleus (13). Here, the viral genes are expressed, viral genomic DNA is synthesized and progeny virions are assembled. The full length DNA must be packaged into these capsids and the capsids on their way out of the nucleus acquire, at least temporarily, a nuclear derived envelope. There is uncertainty about how nucleocapsids become enveloped although no fully enveloped particles are observed in the nucleus (1). There are three possible cytoplasmic routes taken by viral nucleocapsids postulated by Severi *et al* (27). The nuclear-derived envelope is lost, the naked viral particle may go through the Golgi, egress directly through the plasma membrane with acquisition of an envelope or end up as a dense body. If it goes through the Golgi, the capsid becomes sequestered in a vacuole that can (1) fuse with the plasma membrane and be released like a secretory product (2) fuse with a lysosome or (3) fuse with a dense particle.

In fact, three types of viral particles are produced during a productive infection: (1) the regular, enveloped, HCMV genome-containing infectious virion; (2) **dense bodies,** which are enveloped particles that contain nothing but lower matrix protein and (3) enveloped, non-infectious particles which are similar to infectious virus but which do not contain any DNA (1). The production of the latter two types of particles increases with high multiplicity of infection (MOI) *in vitro.*

I.5.1 CELL CHANGES

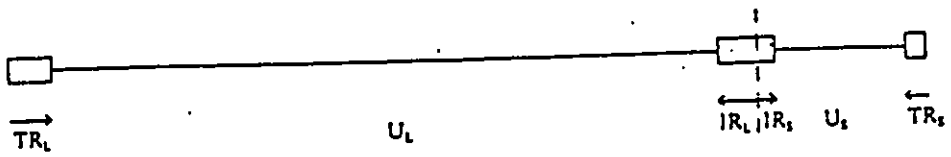
A productive HCMV infection of susceptible cells stimulates their protein and DNA synthesis mechanisms (1). HCMV infection also causes a number of cellular morphological changes and can induce expression of other cellular proteins. These include condensation of chromatin, rounding and enlargement of cells, the presence of nuclear and cytoplasmic inclusions, which is one of the hallmarks of HCMV infection, and induction of cell surface expression of Fc receptors for IgG (28) as well as MHC Class I (29). The induction of MHC expression is not due simply to the effects of interferon produced by infected cells because the enhancement of expression still occurs, albeit to a lesser degree, in the presence of anti-interferon antibodies in tissue culture (29).

I.6 GENOME ORGANIZATION

The genome is divided into 2 components that are separated and flanked by internal and terminal repeat sequences (16, Figure 1). There is a unique long (U_L) segment of about 167 kb and a short unique component (U_S) of about 36 kb. The junction between the long and short components is composed of internal repeat (IR) sequences, which are designated IR_L and IR_S. In addition, each end of the genome is flanked by terminal repeat sequences, TR_L and TR_S.

The HCMV genome is capable of undergoing isomerization. That is, during replication, the L and S components can invert with respect to each other such that 4 different orientations are possible and are found in equimolar amounts in infected cells *in vitro* (14).

FIGURE 1. DIAGRAM OF HCMV GENOME (FROM REF. 14). The genome is 230 kilobases long and is composed of double stranded linear DNA. The boxes represent the terminal (TR) and internal (IR) repeat sequences. The subscript L and S denote the long and short segments of the genome, respectively. The long and short horizontal lines representing the unique long (U_L)and short (U_S) coding regions, respectively. The vertical dashed line highlights the L-S junction which is a hotspot of variability and has been used to distinguish between clinical isolates (30).


TR_L
U_L
IR_L IR_S
U_S
TR_S

This is in contrast to animal CMVs which do not contain internal repeat sequences and so cannot undergo isomerization (13).

The size of the repeat sequences can be quite variable from one clinical isolate to another (30). During replication, both the internal and terminal repeat sequences can amplify resulting in many duplications of the sequence. The junction between U_L and U_S repeat is a major site of interstrain variation (1, 30) due to the variability in size. This region can therefore be used to differentiate between clinical isolates (30).

HCMV DNA usually contains a short sequence designated the 'a' sequence that is present at both viral termini and is found in inverted orientation at the junction between the short and long unique segments (14). It is this sequence that is sometimes amplified to tandem repeats and is flanked by additional short repeat elements and it has been suggested that it may act as a cleavage/packaging signal for the monomerization of virion DNA from concatameric precursors that are produced during viral replication (14).

I.7 VIRAL GENE EXPRESSION

As with all herpesviruses, transcription and translation of the HCMV genome is tightly controlled and regulated in a temporal manner. By the use of various metabolic inhibitors that inhibit translation or viral DNA synthesis, it was shown that there are three basic classes of genes of HCMV based on the time they are expressed during the course of an infectious cycle (14). They are called Immediate Early (IE), Early (E) and Late (L). Furthermore, by examining the function of each class of protein product, it seems that most IE proteins are

regulatory proteins while E proteins tend to be enzymes involved in replication and L proteins tend to be structural proteins. A description of each class follows.

I.7.1 IMMEDIATE EARLY

This genes are of extreme importance because they code for regulatory proteins that turn on and off expression of other viral and possibly cellular genes (31). Following entry of the virus into the cell, a restricted amount of transcription of the HCMV genome takes place. Only the IE genes are transcribed as their transcription does not depend on *de novo* synthesis of viral proteins, unlike the later classes of genes. Following production of these proteins, extensive transcription of the genome can take place.

IE transcripts can be identified by treating infected, permissive cells with cycloheximide or anisomycin which inhibit protein synthesis and leads to the accumulation of IE mRNA (14). There are three IE transcription regions (1): one in U_L at 0.24 map units (mu) of the prototypic genome and one at 0.86 mu in U_S. Sequence analysis of the former shows that it codes for a potential membrane glycoprotein (32) but the transcription of both regions is very low compared to the third region. The functions of the proteins coded for by these two regions have been recently implicated in the regulation of gene expression as well (33). The highest amount of IE transcriptional activity occurs in the third region at 0.66-0.77 map units in U_L with 88% of viral IE mRNA hybridizing to this region and comprising 0.6% of total infected cell RNA (34, 35). This particular region is known as the **major immediate early (mIE)** region. There are two major transcription units—IE1 and IE2— within this region. Both are under the control of the same promoter regulatory region (14).

The IE1 transcript is 1.95 kb in size and is the predominant mRNA produced at immediate early times. It consists of a 5' leader exon plus 3 additional exons (31, 36). The major open reading frame (ORF) actually begins in exon 2 and produces a protein of 491 amino acids that is highly phosphorylated with a molecular weight of 72 kDa. By an alternate splicing mechanism, exons 1, 2, and 3 can be ligated to exon 5 to produce the IE2 transcript. Most of the IE2 protein is encoded by exon 5 and produces a protein of 55 or 86 kDa depending on whether an in-frame intron is removed or not (1). Both proteins share 85 amino acids from exon 2 and 3 and both are detected almost exclusively in the nucleus, consistent with their role as transcriptional regulatory proteins (37).

Many *in vitro* studies have been done using transient transfection assays employing homologous and heterologous inducible promoters linked to reporter genes to demonstrate the IE proteins' regulatory effects. The picture that emerges is that they have both cooperative and opposing effects. For instance, the IE1 protein has been shown to enhance the expression of some HCMV early gene promoters (1) as well as its own expression. Since IE2 is transcribed from the same promoter, its expression is also enhanced as a result of IE1 transactivation (1). IE2 can transactivate early promoters as well and both IE1 and IE2 can synergize to transactivate early promoters to a much greater degree than either one alone (38). In addition to transactivation, the IE2 protein is also capable of repressing expression from the major immediate early promoter (38).

The effects of these proteins are not limited to HCMV viral promoters. Studies have shown that the immediate early proteins can influence the expression of cellular (39-41) as

well as other viral promoters including HSV, adenovirus (41, 42) and HIV (43).

In summary, then, the immediate early proteins are the first to be expressed on entry of the virus into the cell. Their function essentially is to turn on the expression of other genes, most notably the early viral genes such that the infectious cycle can continue.

I.7.2 EARLY PROTEINS

Beginning around 2 hours post-infection, the early genes begin to be transcribed (1). Their expression is dependent on the IE proteins. These E gene transcripts were initially identified by blocking viral DNA synthesis. Once turned on and once viral DNA is being produced, abundant transcription takes place throughout infection (1).

Most of the early proteins are enzymes involved in viral DNA replication. The HCMV DNA polymerase is one good example of such an early protein. It shows 24% amino acid homology with the HSV polymerase (14).

Other enzymes are assumed to exist based on sequence analysis of the AD169 genome. A ribonucleotide reductase, a helicase, a deoxy-UTPase and a DNAse have all been predicted to be coded for by HCMV (12).

I.7.3 LATE PROTEINS

The transcription and translation of the late genes start prior to viral DNA replication, but maximum expression occurs only after replication has begun (44). How transcription of the late genes is regulated, however, has not been well characterized. The IE proteins probably play a role (45) but post-transcriptional events are probably involved as well since the transcription of some late genes occur early, but high levels of protein are only seen late

in infection (45, 46). The late proteins are essentially structural proteins found within virions. These include the envelope glycoproteins, matrix proteins and capsid protein.

I.8 HUMAN IMMUNE RESPONSES TO HCMV

Considering the complexity of the virus, it is not surprising to find that immune responses are generated against many different HCMV proteins. Both antibody (Ab) and cell mediated responses have been studied.

Studies of the humoral immune response in humans have shown that as many as 15 to 20 HCMV proteins are capable of inducing antibody to varying degrees (47-49). The question then becomes which of these responses are important, if at all, in helping to resolve an active infection and to help prevent a reactivation or superinfection.

In terms of the intensity of responses against HCMV proteins, the most strongly reactive HCMV proteins in immunoblot and immunoprecipitation assays are gp58 (a glycoprotein) and the matrix proteins—pp150, pp65 and pp28 (50, 51). The significance of antibodies against matrix proteins is not clear. One can imagine that virus neutralizing antibodies would be more important in resolving infection by helping to prevent viral spread. The envelope glycoproteins are perfect candidates in this regard and in fact, neutralizing antibodies are generated, both complement dependent and independent, against all three glycoprotein complexes (17, 52, 53). The generation of monoclonal antibodies against these proteins have been particularly useful in elucidating which proteins are being recognized by antibodies.

Cytomegalovirus infection is probably controlled more by the action of HCMV antigen specific cytotoxic T lymphocytes (CTLs) as opposed to the effects of neutralizing antibody. This conclusion is supported by the observation that the ability to mount a specific CTL response against HCMV is inversely correlated with the spread of virus in the host, whereas no such correlation has been found between resolution of HCMV disease and HCMV specific antibody titers (17, 54). It is when the cell mediated immune responses are suppressed, either on purpose to prevent organ transplant rejection or are deficient as in AIDS patients, that one finds symptomatic and sometimes severe HCMV infections even in the presence of anti-HCMV antibodies.

The majority of the CTL response, as judged by the generation of human CTL clones, appears to be directed against the 72 kDa IE1 protein (55, 56). This would be a logical target for an immune response, as it is the first protein to be expressed in infected cells before the generation of new infectious virus. Eliminating the cell at this point could possibly prevent spread of the virus. Another report, however, came to a different conclusion and found that the majority of the CTL response is generated against a structural virion protein (57) with viral gene expression not being required for CTL recognition. While it is clear that CTL responses are important in controlling HCMV infection, it is not so clear which is the immunodominant antigen.

I.9 THE EFFECTS OF HCMV ON THE IMMUNE SYSTEM

HCMV's interaction with the immune system is not unidirectional. That is, while the immune system is able to respond against the virus, HCMV has the capability of suppressing certain immune responses during an active infection. Studies of peripheral blood lymphocytes

from patients with HCMV mononucleosis have shown depressed *in vitro* responses to various mitogens such as concanavalin-A, pokeweed mitogen and phytohemagglutinin as well as diminished proliferative responses to other herpesvirus antigens (58). Other findings include depressed CTL (59) and Natural Killer functions (60) *in vitro* although antibody responses appear to be normal. In addition, the normal CD4 to CD8 T cell ratio is inverted during an active infection which is due to an increase in CD8 positive T lymphocytes (58). The cellular responses as well as T cell ratios do return back to normal during and after the convalescent phase of HCMV illness (58).

I.10 EPIDEMIOLOGY

HCMV is a prevalent virus in most geographical areas. Exposure to HCMV, as judged by the presence of anti-HCMV antibodies in serum, ranges from 40-100% depending on the population or subpopulation being studied and roughly correlates with socioeconomic status, the number of sexual partners and type of sexual activity (61, 62). In particular, homosexuals have a very high prevalence rate of HCMV Ab often close to 100%. By age 35, for example, 95% of homosexual men in the USA have antibodies to HCMV (63). The prevalence increases with age from childhood to adulthood probably due to sexual spread.

HCMV has been isolated from many body fluids including saliva, vaginal secretions, semen, urine and breast milk (63). The uterine cervix can be infected as well. The virus is spread by close, intimate contact including sexual intercourse both vaginal and anal, and by blood transfusion and organ transplant (63). Transplacental and perinatal acquisition of virus

is also possible (63). Transmission in the day care setting from child to child and child to day care worker can potentially spread the virus as well (64).

One of the reasons why HCMV is highly prevalent is probably because of its sexual transmission and the fact that HCMV is shed into oral and genital secretions asymptomatically many months after an acute infection. In a report (62) of homosexual men that were studied longitudinally, for example, HCMV was excreted in semen more consistently than in urine and for a longer period of time—an average of 22 months for semen and 9 for urine. From this study, it was also shown that most HCMV seropositive homosexual men excrete cytomegalovirus at least intermittently and that this shedding was inversely correlated with age. That is, younger individuals tend to excrete HCMV more often than older individuals.

I.11 HCMV AND CELL TROPISM

One of the big questions that still remains to be satisfactorily answered is which cell types can be infected by HCMV and which cell type(s) is the main reservoir of latent virus? *In vitro*, the only cell type that yields infectious virus and that is used routinely is the human diploid fibroblast (24). Even in these cells the virus grows so slowly that it can take six weeks to get a positive culture result (65). This cell, however, is not the typical infected cell found *in vivo* (1). Of particular interest is which white blood cell can be infected as it is known that blood transfusion is a source of virus (61) even though attempts to culture HCMV from blood from normal, seropositive donors have been, for the most part, unsuccessful with only one reported success from 2 out of 35 asymptomatic donors in 1969 (66). The presumption here

is that HCMV is present in a latent form and this latency is maintained in *in vitro* culture techniques or that the amount of latent virus is so small that it is not detectable by culture. It is also important to define which cell type(s) are infected because of the possible interactions between HCMV and HIV that may accelerate HIV disease progression (67). This will be discussed in a later section.

Many studies have been published reporting the detection of HCMV in a variety of blood cell types. When reviewing the literature on the subject, it is important to keep in mind the source of blood cells for the study—be it from an actively infected or latently infected donor—and the viral detection system used because the sensitivity of detection of different systems substantially differ. These variables will influence the results obtained and can help explain the discordant results that are sometimes reported.

There is little dispute that during an active, symptomatic infection by HCMV, it is not at all difficult to culture HCMV from blood and other tissues (65, 68). Most of the time the buffy coat of blood, which contains all of the white blood cells, yields infectious HCMV in culture and it has been reported that polymorphonuclear leukocytes (PMNL) are the greatest source of virus during an active infection (68-70). Virus can be cultured from mononuclear cells as well, although less consistently and the titer of virus is apparently lower (71, 72). When HCMV DNA hybridization (69) or immediate early proteins detected by immunofluorescence (73) are used to detect virus instead of by culture, HCMV is consistently found in mononuclear cells and PMNLs, although again, more HCMV is found in the PMNL fraction.

The presence of HCMV in PMNLs has been hypothesized to result from phagocytosis by the PMNLs of intact virions or to arise as a result from infection of progenitor cells in the bone marrow (71) suggesting that HCMV replication does not take place in PMNLs. However, Dankner *et al* (70) detected HCMV mRNA by in-situ hybridization from all temporal gene classes indicating that perhaps HCMV does replicate in polymorphonuclear leukocytes.

In terms of which lymphocyte can support HCMV replication, early studies in which peripheral blood lymphocytes were cultured from renal transplant patients (74) or in which lymphoblastoid cell lines were infected *in vitro* and HCMV DNA (75) or whole virus (76) replication were monitored have suggested that T and B cells support very little, if any, HCMV replication. Only 3 out of one million infected T cells, for example, were found by Garnett to be positive (74) and less than 1% of cells produced infectious virus in another study (76). Only 0.05% of peripheral blood T cells supported HCMV AD169 virus replication (77). A fundamental limitation of these studies (75-77) was the fact that laboratory strains of HCMV—which have adapted to grow well in fibroblast cells (78) and not other cell types—were used, so it is not necessarily surprising to find such small amounts of virus in these other cell types.

Turning to the search for latent virus *in vivo,* sensitive molecular biological techniques had to be employed to detect HCMV. Two publications are of particular interest here: first, Rice *et al* (79) found that most peripheral blood mononuclear cell types—from blood donors of undetermined HCMV serostatus—were capable of being infected to different degrees as

shown by detection of immediate early gene products with monoclonal antibodies in immunofluorescence assays. The interesting observation was that only the immediate early gene products were being expressed—no infectious virus was produced. Monocytes had the greatest proportion of cells expressing the IE gene products. Secondly, using radiolabelled cloned portions of the HCMV genome in *in situ* hybridization studies of cells from seropositive, asymptomatic individuals, Schrier *et al* (80) found HCMV DNA in 0.03-2% of peripheral blood lymphocytes. Infectious virus could not be produced from these cells. Schrier *et al* did not look at monocytes but found that the majority of positive lymphocytes were CD4 positive. Other studies using the polymerase chain reaction to detect HCMV DNA in peripheral blood (81, 82) further substantiate the presence of HCMV DNA in mononuclear cells of seropositive, asymptomatic individuals with Taylor-Wiedeman *et al* (82) demonstrating that HCMV was detected predominantly in monocytes.

With monocytes being increasingly implicated as an important source of HCMV, a recent paper (83) showed that monocytes can harbour HCMV. In addition, they also supported virus replication when they differentiated into macrophages *in vitro.* The macrophages do not appear to suffer any cytopathic effects. What we are left with is the intriguing possibility that monocytes may be reservoirs of HCMV and that under appropriate conditions they produce new infectious virus. Should this occur *in vivo,* this may be a mechanism in which the virus can persist and spread throughout the body.

Finally, in terms of other cell types infected, HCMV has been detected in the epithelial and endothelial cells of many different organs including the kidney, liver, salivary gland and

in glial cells and neurons (84, 85). In disseminated infections, the virus can be found in almost every organ that has been examined (86).

I.12 HCMV: THE PROBLEM

In individuals with competent immune systems, HCMV does not cause life-threatening disease. Primary infection is asymptomatic in 90% of individuals (87) but a mononucleosis-like disease can occur with symptoms of fever, abnormal liver function, peripheral blood lymphocytosis, atypical lymphocytes, and transient immunological abnormalities (71) which can be distinguished clinically from EBV-mediated mononucleosis by the absence of pharyngitis, for example (88). The illness eventually resolves and the individual becomes an asymptomatic carrier of the virus.

There are three subpopulations of individuals, however, in which HCMV can cause life-threatening illness and each of these groups will be briefly described: neonates, transplant recipients and AIDS patients—basically any individual whose immune system is somehow compromised or suppressed.

I.12.1 *IN UTERO* AND PERINATAL INFECTIONS BY HCMV

HCMV infects about 1% of all babies born in the USA (61). It is the most common cause of congenital and perinatal viral infections throughout the world (61) and it is the most common viral cause of mental retardation and nonhereditary sensorineural deafness (89). Congenital versus perinatal transmission in an asymptomatic baby can be distinguished based on the culture of HCMV from urine or saliva in the first week of life (90). Of the 1% of

babies with HCMV, roughly 90% are asymptomatic. Of the 10% who are symptomatic, one half of these have typical cytomegalic inclusion disease (CID), the other half have atypical involvement (91). 90% of the symptomatic babies develop severe debilitating complications such as mental retardation and hearing loss with a mortality rate of about 30% (61). The involvement of multiple organs can occur. Hepatosplenomegaly, microcephaly, jaundice, intrauterine growth retardation and prematurity are potential complications (1).

A small percentage (1-15%) of asymptomatic newborns are at risk for developmental abnormalities like sensorineural hearing loss, microcephaly, motor defects, mental retardation, and dental defects which usually become apparent within the first 2 years of life (61).

In perinatal infections, transmission of HCMV is thought to occur by transmission of HCMV through breast milk or by ingestion of cervicovaginal secretions during birth (91). In the United States, 1-15% of infants are perinatally infected by 6 months of age (91). Infection is associated with persistent shedding of virus in urine and saliva for years although at a lower titer versus congenital infections (91). Most perinatal infections are asymptomatic.

I.12.2 TRANSPLANT RECIPIENTS & HCMV

Transplant recipients are also susceptible to potentially life-threatening HCMV infections and it represents the single most important infectious agent affecting recipients with at least 2/3 having HCMV infection one to four months post transplant (92). Transplant patients must be iatrogenically immunosuppressed in order to minimize the risk of graft rejection. Unfortunately this can allow HCMV to reactivate in those recipients who are seropositive or it allows for serious primary infections of seronegative recipients most of

whom appear to be infected from the donor organ rather than from blood transfusions (92), both of which can transmit HCMV. In kidney transplants, for example, the source of HCMV infected cells is the donor kidney in 80-90% of the cases (92-95) with the remaining infections occurring from blood cells. Superinfection with another strain of HCMV is also possible as shown by molecular epidemiological studies (93-95).

The incidence of symptomatic disease, however, depends on the type of infection as just described: 2/3 of primary infections result in disease and between 20-40% for reactivation or superinfection (92). The symptoms can be quite severe, including HCMV pneumonia, fever, hepatitis, colitis and perhaps, indirectly, graft dysfunction (92).

I.12.3 HCMV IN HIV POSITIVE INDIVIDUALS

Finally, there is HCMV infection in the setting of HIV infection. In North America, the prevalence of HCMV is quite high in adult populations and is even higher (up to 100%) in the homosexual community, which has the highest prevalence of HIV infection. In fact, before HIV was first identified, it was thought that HCMV might even be the etiologic agent of AIDS because of its ubiquity and its immunosuppressive capabilities (96-98).

HIV causes immunodeficiency due to the progressive loss of CD4 positive T cells (99), which are of central importance in mounting immune responses. Thus, with the progressive loss of an effective immune response, HCMV can reactivate in HIV infected individuals and frequently does. One finds a progressive increase in HCMV viremia as HIV disease progresses (100, 101). Here again, significant morbidity can occur including sight-threatening HCMV retinitis and HCMV infections of the gastrointestinal (GI) tract and

perhaps the lung (101). Autopsy and clinical studies indicate that 90% of patients with AIDS develop an active HCMV infection during their illness and up to 40% may experience sight or life threatening infections (87, 103). They have also shown that disseminated HCMV infections are a frequent occurrence but are not always obvious. In one report, for example, 14 out of 15 AIDS patient autopsies revealed the widespread presence of HCMV in the lungs and adrenal glands (104) and in many other organs (86, 105-107). The frequency of HCMV retinitis as an initial AIDS defining opportunistic infection was 2.4% in 1989 (108) in the United States.

In addition to the overt morbidity associated with HCMV infection in AIDS patients, another area of great concern is the possibility that HCMV and HIV may interact at the cellular and molecular level, with this interaction resulting in the enhancement of virus replication (67, 109) and allowing for a more rapid progression to AIDS. Some have evoked the concept of a cofactor to explain why there is such a long latency period between acquisition of HIV and development of AIDS, which can be 10 years or longer (67). That is, there might be some other factor in addition to HIV which is required to drive the progression to AIDS.

How might such a relationship exist between HCMV and HIV? Several hypotheses exist, none of which are mutually exclusive. The first is simply that an immune response directed against HCMV stimulates the production of cytokines that may activate the expression of HIV (67). *In vitro,* supernatants from monocytes stimulated with HCMV proteins increased the production of HIV in chronically HIV infected cells whereas other

herpesviruses, adenovirus or vaccinia virus could not (107). Additive immunosuppression is another possibility (67, 111). Since HCMV can be immunosuppressive as well, the combined effects of both viruses on the immune system might allow a greater degree of viral replication.

Another potential mechanism might be that HCMV allows HIV to infect cells for which it is not normally tropic (67). Such a phenomenon has been demonstrated *in vitro* (28) whereby fibroblasts were induced to express Fc receptors for IgG as a result of HCMV infection. This allowed the uptake of HIV coated with IgG antibodies. Although no *in vivo* evidence exists that such a process occurs, it may be a way by which the two viruses can coinfect the same cell. Coinfection of the same cell is potentially a big problem, as it has been demonstrated in many studies that HCMV is capable of transactivating the HIV via its long terminal repeat (LTR) in a variety of cell types (43, 112-117). It is the immediate early proteins of HCMV—which are necessary for regulating the expression of its own genome—that are responsible for the transactivation of the HIV LTR. The stimulation of viral expression is not only limited to HIV, as transactivation by the HIV tat protein of HCMV gene expression has been demonstrated to occur *in vitro* (109, 118). But the transactivation of HIV is clearly dependent on cellular factors as well because in some cell types transactivation does not occur and inhibition of HIV replication might even occur (111).

In terms of *in vivo* evidence of an interaction, studies have focused on following a cohort of HIV infected individuals with known dates of HIV seroconversion and comparing the percentage of individuals who progress to AIDS between those that are seropositive for HCMV—and thus have been exposed to the virus and are presumably latently infected by it—to those that are HCMV seronegative. In one study of a hemophiliac cohort, for instance,

over a 9 year period, 41% of HCMV seropositive patients had progressed to AIDS while only 13% of HCMV seronegative patients had done so (119). The relative risk of developing AIDS was calculated to be 2.5 for HCMV seropositive individuals. No association was found between the presence of HSV and HIV. A follow up report two years later (120) confirmed their earlier findings. No other study has reported data that relate HCMV seropositivity to progression to AIDS after HIV seroconversion so further studies are needed to confirm these observations.

I.13 DETECTION OF HCMV INFECTION

Due to its importance as a pathogen in the above patient populations, a rapid, sensitive, reliable test is required to detect the presence of HCMV. Currently, there are a number of methods available, all with their own advantages and disadvantages.

The so-called "gold standard" is the cultivation of the virus from a tissue, body fluid or blood specimen. It is ideal to see virus actually growing from a specimen rather than detecting the virus in an indirect manner. The problem here is that HCMV grows extremely slowly *in vitro*. It may take up to six weeks to see any cytopathic effect with an average of 1-2 weeks (65). This clearly is not fast enough.

Electron microscopy has also been used (121) to directly visualize the virus in a clinical sample. The problem is that the technique lacks sensitivity, the virion structure of HCMV is not unique and may be confused with other herpesviruses (122) or other viruses like adenovirus which may also produce intranuclear inclusions like those of HCMV. There

are several serological techniques that detect antibodies (IgG or IgM) to HCMV: for example. the Enzyme linked Immunosorbent Assay (ELISA), immunofluorescence assays, latex agglutination assays etc. These assays are very useful in epidemiological surveys to determine who has been exposed to HCMV and who has not; for screening of blood and organ donors in order to determine potential for transmitting latent HCMV (65); and to determine primary infections. Their disadvantage is that it is difficult to diagnose a secondary or reactivated infection unless serial serum samples are available to demonstrate a rise in Ab titers and not all of the Ab tests are licensed for this purpose. In general, problems with Ab tests include nonspecific reactivity and a lack of sensitivity (65) and in IgM assays, interference by rheumatoid factor and competition for antigen (Ag) by IgG are always potential problems (65) although techniques are available to decrease this interference.

Immunocytochemical techniques, such as immunofluorescence have also been employed. This involves staining potentially infected cells with a fluorescent Ab typically against the 72 kDa IE protein and fluorescent cells indicate the presence of HCMV. A variant of this technique, the shell vial assay, is available (123). This procedure combines culture with immunofluorescence. A specimen is centrifuged onto fibroblasts which is thought to increase adsorbance of virus onto permissive cells (123). Combined with fluorescent Ab staining, this decreases the time it takes to perform the test to as little as 18 to 72 hours (123, 124). Care here must be taken to ensure the cells are viable as many commercial sources of shell vials have a limited shelf life (125). A poor quality vial results in decreased sensitivity of detection and increases the susceptibility of cells to toxic effects of urine, for example, and thus increasing the chance of a false negative result (125).

In the era of molecular biology, research has focused on recombinant DNA technologies as a means of detecting virus. Cloned portions of virus labeled with radioactive isotopes have been used in DNA dot blot, *in situ* hybridization assays and Southern blots to detect viral DNA sequences in specimens (69, 70, 121). The technique has the capability of detecting as little as 5 picograms of HCMV DNA which corresponds to about 10^4- 2 x 10^5 genomes (124). The problem is that the vector used to clone the HCMV DNA may have homologies with bacterial DNA sequences (126) and certain HCMV gene sequences are homologous to cellular DNA (127, 128). So false positive results are a potential problem unless great care is taken in choosing a unique HCMV sequence and in purifying the cloned DNA from its vector. The expense and potential biohazard risk associated with the use of radioisotopes is another area of concern.

An alternative to the above tests has recently been described. The polymerase chain reaction has revolutionized molecular biology and is increasingly employed as a diagnostic tool for infectious diseases. As with all procedures, it has its advantages and disadvantages which will be explored in the next several sections.

I.14 THE POLYMERASE CHAIN REACTION

PCR is a molecular biological technique in which a stretch of DNA is copied or amplified many fold. It was "discovered" in 1985 by Kary Mullis (129) although all of the components for the reaction had been available for many years. But it was Kary Mullis who thought of putting them all together. The first PCR paper was published in 1985 (130).

The technique is conceptually quite simple: one first identifies the sequence that one wants to amplify by designing 2 oligonucleotide primers—which are short, single stranded DNA molecules—that delineate the sequence of interest. One primer, the upstream forward primer bonds to one strand of the double stranded DNA molecule while the reverse or downstream primer bonds to the other strand. Copies of the molecule can then be made by adding the appropriate building blocks of DNA: deoxynucleoside triphosphates (dNTPs), and a DNA polymerase that catalyzes the reaction in a suitable buffer.

Mechanistically, there are 3 steps: (1) **denaturation,** in which the target or template DNA must be separated into single strands in order that the oligonucleotide primers, present in molar excess, can (2) **anneal** to their respective complementary, specific sequence. Finally, the DNA template is copied by the DNA polymerase in an (3) **extension** step. The product of this reaction is a new double stranded DNA copy of the target sequence. If one repeats this "cycle" of denaturation, annealing and extension 20 or 30 times, the DNA copies can accumulate in an exponential fashion because the copies themselves can become templates in subsequent cycles. Starting, then, from one copy of a DNA target sequence and after doing 30 cycles of PCR one can theoretically end up with a million copies of the sequence.

The initial DNA polymerase used in PCR was the Klenow enzyme from *E. coli* and, before the invention of an automated thermocycler, the reaction was carried out by transferring the tubes into water baths at the appropriate temperatures for each step of the reaction. The problem with Klenow is that it is not thermostable. That is, it denatures at the high temperatures required for denaturation of the template such that in each cycle of the

reaction, new enzyme had to be added. This was a tedious and risky process, given the chance of exogenous DNA contaminating the reaction. This also limited the number of reactions that could be performed at any one time. However, another DNA polymerase was soon substituted for Klenow, the so called **Taq** polymerase (131). This enzyme was originally isolated in 1976 (132) from the thermophilic bacterium *Thermus aquaticus* which was found in the hot springs of Yellowknife National Park in the USA in 1969 (133). The bacterium requires proteins that can withstand high temperatures in order to survive and so it is not surprising that it contains a thermostable polymerase. Indeed, the optimum temperature at which it extends is within the range of 75-80°C (133) and has a half-life at 95°C of about 40 minutes. Its use allowed for several advances in PCR: it allowed an increase in the annealing temperature of the reaction from 37°C for Klenow up to 50-65°C, which allows for greater specificity (134) and it allowed the reaction to be automated since fresh enzyme does not have to be added at each cycle. This, in turn, allowed for the development of the thermocycler, which quickly and automatically changes the temperature for each step of PCR (135). The switch to Taq also increased the upper size limit for amplification. With Klenow, only fragments up to 400 bp could be amplified while with Taq, stretches of up to 10kb can be amplified (135). The enzyme has a specific activity of at least 60 nucleotides per second per enzyme molecule at 70°C (133).

The original purpose of PCR, as envisaged by Mullis, was to aid in DNA sequencing (129). It soon became apparent, however, that many other uses were being developed. For example, PCR has been used to examine nucleotide sequence variation (136-138),

chromosomal rearrangements (139), used for cloning of genomic sequences (140) and for direct sequencing (141) without the need for cloning or PCR product purification and in diagnosing genetic diseases such as sickle cell anemia (130, 137), Duchenne muscular dystrophy (142), and in the detection of viral pathogens like HIV (143).

Variations of the basic PCR technique have also been reported. Here, one hears the terms multiplex PCR (142), asymmetric PCR (144), inverted or inside-out PCR (145, 146) and nested PCR (147, 148), for instance. **Asymmetric** PCR is a method of generating single stranded product instead of the usual double stranded product by using a vast molar excess of one primer over the other. **Multiplex** essentially means doing more than one amplification per reaction. So, for example, one could have 6 different primer pairs in one reaction that amplify 6 different products. In **inverted** PCR, the primers are directed away from each other instead of toward each other such that the regions outside of the primer region instead of between the primers are amplified. This is important if one wants to sequence an unknown region, for example, that surrounds a known sequence. Finally, in **nested** PCR, 2 consecutive amplifications are performed instead of just one. The first amplification is performed with one set of primers and then a small aliquot of this product is put into a second fresh reaction containing primers whose complementary target sequence is contained within the first amplification product. This results in an increase in sensitivity and specificity over a single PCR amplification because of the greater amount of amplification achieved with two reactions and it should theoretically decrease false positives through the use of a second, internal, specific pair of primers. This is the approach that was chosen for our HCMV PCR assay.

I.14.1 METHODS OF DETECTION OF PCR PRODUCTS

Once an amplification is performed, it becomes necessary to verify that DNA has been amplified and, at least initially, that the expected product size (i.e. specific product) has in fact been produced. The easiest way is just to run a portion of the amplification reaction on an agarose or polyacrylamide gel stained with ethidium bromide in order to visualize the DNA. Gel electrophoresis separates DNA fragments essentially by size and so by running a molecular weight ladder or positive control in the gel as well, one can verify that the expected product size has been produced. One can also sequence the DNA product to ensure that the correct sequence has been amplified. This can be done initially but becomes impractical to do routinely.

However, if sensitivity is a concern as it is in infectious disease diagnosis, it is usually necessary to enhance the sensitivity by adding an enhanced detection system. There are at least a couple of ways of doing this, generally either colorimetrically (149) or through the use of radioisotopes (130). So either the primers or an additional oligonucleotide probe complementary to an internal product sequence is labeled with a fluorescent or isotopic ^{32}P marker, for example. These systems allow the detection of a smaller quantity of specific product that is not detectable by ethidium bromide staining. Sensitivity can be increased 10-1000 fold using labels. A probe also has the advantage of providing an extra measure of specificity as well, since it has been designed to specifically hybridize to a sequence contained within the amplified sequence.

I.14.2 OPTIMIZATION OF PCR

A good PCR assay is both sensitive and specific. A sensitive assay is one that detects

even small amounts of the target sequence and a specific assay is one that detects the particular sequence and will not amplify other sequences that may share partial homologies with the primers being used.

The keys to having a good assay are carefully choosing the primers and empirically adjusting both the thermoprofile and reaction components in order to achieve the desired level of sensitivity and specificity. All of the components of the reaction—from the time and temperature of annealing to the concentration of reaction components—affect the quality of the reaction. There is no universal set of optimal conditions for PCR such that for each set of primers, the optimal reaction conditions should be empirically determined although the range of conditions in which PCR works best are well known (133).

The first thing that one has to do is to choose the primers carefully. In order to avoid complementarity to a homologous sequence that may be randomly present in the human genome, for example, the primers must be at least 17 nucleotides long (134). To make sure that they are not homologous to other known sequences, the chosen primers can be screened through sequence databases such as GenBank or other such sequence database. The primers should also stably anneal to the target sequence (150) and should not be internally complementary nor should they be complementary to each other. Complementarity to each other, especially at their 3' ends, results in the formation of primer-dimers—the annealing of primers to each other. This can significantly decrease the amount of primer available to bind to the target sequence thus reducing the yield of product. Primer concentration is another

consideration. Too high a primer concentration can result in non-specific product formation with specific product yield again being reduced.

After the primers have been selected, the reaction components must then be adjusted. In terms of the thermoprofile, what typically changes here are the number of cycles, and the time of each step as well as the temperature of annealing. The denaturation and extension temperatures are more or less standard—94-95°C and 70-72°C, respectively, are most common. The time at which they are held at these temperatures is dependent on product size with larger products requiring longer times, especially for extension. The annealing temperature has a great affect on specificity of the assay. At low temperatures (less than 50°C, for example), non-specific annealing can occur resulting in amplification of non-target sequences because mismatches between primer and template can be tolerated except at the 3' end where the Taq extends from the primer. As the temperature of annealing increases, hybridization of the primer to target becomes (135) more stringent as mismatches between the primer and template become more and more unstable such that only perfectly matched species anneal and are extended. The time of extension of primers also affects the specificity of Taq.

Even with a perfect set of primers, non-specific products can still accumulate as specificity of PCR is also determined by the concentration of reaction components, including the initial target concentration, and the number of cycles the PCR is carried out (134). The amount of Taq, magnesium and dNTPs all have an effect. Usually, an excess or deficit of a component results in an increase of spurious products.

Once the PCR has been optimized, usually done with control reactions, the assay is then assessed in order to determine its effectiveness in the screening of clinical samples.

I.14.3 PCR: THE PITFALLS

The high sensitivity of PCR is one of its greatest advantages yet it is also its greatest weakness (135). Contamination of a reaction with a target sequence from a previous reaction can lead to a false positive result. Fortunately, there are a number of well-established procedures that can be implemented so as to reduce this occurrence. These procedures are almost always outlined in every review of PCR, most of which are implemented here. For example, the use of positive displacement pipettors and aerosol resistant tips to prevent contamination of pipetting equipment with DNA; to use different, dedicated pipettors for sample processing, PCR and post PCR manipulations and to use separate areas for these purposes. Newer, commercial techniques include the use of a PCR carry-over prevention kit from Perkin Elmer that enzymatically chews up previous amplification products that contain deoxyuracil or the use of isopsoralen compounds and UV irradiation to cross link DNA such that it can no longer serve as an amplifiable target.

I.14.4 PCR: APPLICATION TO HCMV

PCR has been and will continue to be examined as a diagnostic tool for detecting both genetic and infectious diseases. It was my intention to investigate the application of PCR to the detection of HCMV.

The area of the genome that was chosen for amplification was the major immediate early gene region. Extreme care must be taken when choosing primers for the amplification

of HCMV because certain areas of the genome are known to be homologous to both human sequences (127, 128) and to HHV-6 (151). These genomic areas must be avoided for the sake of specificity. Another concern is that of HCMV genomic variability. If being used as a diagnostic procedure, then an assay that amplifies all clinical strains is essential. Little is known about the genomic variability of HCMV among clinical isolates except that restriction enzyme patterns can be different from isolate to isolate (152, 153) and that the L-S junction is a hotspot for variability (30). Again, areas of high or suspected areas of high variability should be avoided. In other published HCMV PCR assays (eg. 90, 124, 154) that have used the IE gene region, all isolates were detected suggesting that this genomic region is not highly variable making it a good target for PCR assays. Given that the IE gene region codes for regulatory functions essential to begin viral replication, it is perhaps not surprising that the IE genes would be well conserved.

For this work, two fully nested primer sets were developed for amplification of IE genes. One set (CMV set) starts in exon 2 and ends in exon 3 while the second set (DEM) resides entirely within exon 4. A further description of the primers can be found in *Materials and Methods*.

I.15 OBJECTIVES

As stated, HCMV can cause significant morbidity and mortality in neonates, transplant recipients and AIDS patients. A reliable, rapid, sensitive and specific test is essential in order to diagnose and treat HCMV disease and to monitor the response to anti-viral therapy.

The objectives, then, were (1) to develop and characterize two PCR assays for HCMV DNA which involved the selection of primers, the development and optimization of nested PCR for HCMV, and to verify the sensitivity and specificity of these assays. Secondly, to determine if PCR can be used to detect clinically relevant HCMV viremia as opposed to detecting HCMV in an individual who does not have overt HCMV mediated disease. This involved the use of PCR in HIV negative blood donors of known HCMV serostatus, in HIV seropositive individuals who do not have AIDS and in individuals with AIDS with and without known HCMV disease.

II. MATERIALS AND METHODS

II.1 CLINICAL SAMPLES

99 samples from 28 different individuals were tested. They were separated into 3 groups: (1) 14 individuals who were HIV-1 seronegative; (2) 5 asymptomatic HIV-1 seropositive individuals who had not progressed to AIDS; (3) 9 individuals with AIDS. HIV-1 infected individuals were classified according to the CDC classification system (155).

II.2 ISOLATION AND LYSIS OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMCs were isolated from whole venous blood by centrifugation through a Ficoll-Hypaque (Pharmacia) gradient, washed twice in PBS and adjusted to a concentration of either 3×10^6 or 6×10^6 cells/mL in lysis buffer (10 m*M* Tris-HCl [pH 8.3], 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P-40). The samples were incubated with proteinase K (Sigma) at a concentration of 60 µg/mL for 1 hour at 56°C followed by proteinase K inactivation at 95°C for 10 minutes. Prior to using the sample for PCR, the cell lysates were boiled in order to denature the DNA for 10 minutes followed by quenching on ice for 5 minutes. Processed samples were stored at -70°C.

II.3 CONTROL CELLS FOR PCR

As positive controls for PCR and in experiments to determine the sensitivity of the PCR assays, MRC-5 human fibroblasts infected with HCMV strain Davis (American Type Culture Collection, ATCC) were used.

In order to establish the specificity of the PCR assays, cells infected with the other herpesviruses were tested. These included HSV-1 strain F, HSV-2 strain G, VZV strain Oka, all of which were purchased from ATCC and used directly without culturing; GM-06818—an EBV transformed B cell line (Coriell Institute for Medical Research) and HHV-6 infected cord blood mononuclear cells provided by Dr. F. Diaz-Mitoma (Children's Hospital of Eastern Ontario, Ottawa). The cells were thawed, washed twice in PBS and resuspended in lysis buffer.

PBMCs isolated from Red Cross blood donor units were used as a source of human, HCMV negative DNA. All of the cells were lysed in the same way as just described above.

II.4 OLIGONUCLEOTIDE PRIMERS FOR PCR

All of the primers were synthesized at the Regional DNA synthesis laboratory of the University of Calgary on a Model 394 DNA synthesizer (Applied Biosystems, Foster City, CA) using methoxyphosphoramidite chemistry. The crude oligonucleotide product was purified by polyacrylamide gel electrophoresis.

Three sets of nested primers were evaluated for use in the PCR. The sequences of these primers as well as their nucleotide position within the immediate early gene region are

provided in Table 1 and Figure 2. The nucleotide sequence numbers are as per Akrigg *et al* (36). The first nested set , the MIE set, has its outer forward and reverse primers in exons 2 and 4, respectively, with the inner or nested MIE primers in exon 3. The next set, named CMV and which are a partial derivative of the MIE set, similarly has outer forward and reverse primers (OF and OR, respectively) as well as the nested forward and reverse primers (INF and INR).Finally, the third set is called DEM with the same primer nomenclature as the CMV set to identify outer and inner forward and reverse primers. CMV-OF is in exon 2 while CMV-OR, INF and INR are in exon 3 of the major immediate early gene region. All of the DEM primers reside in exon 4. The size of the amplification products are provided in Table 1.

II.5 STANDARDIZED PROTOCOL FOR HCMV PCR AMPLIFICATION

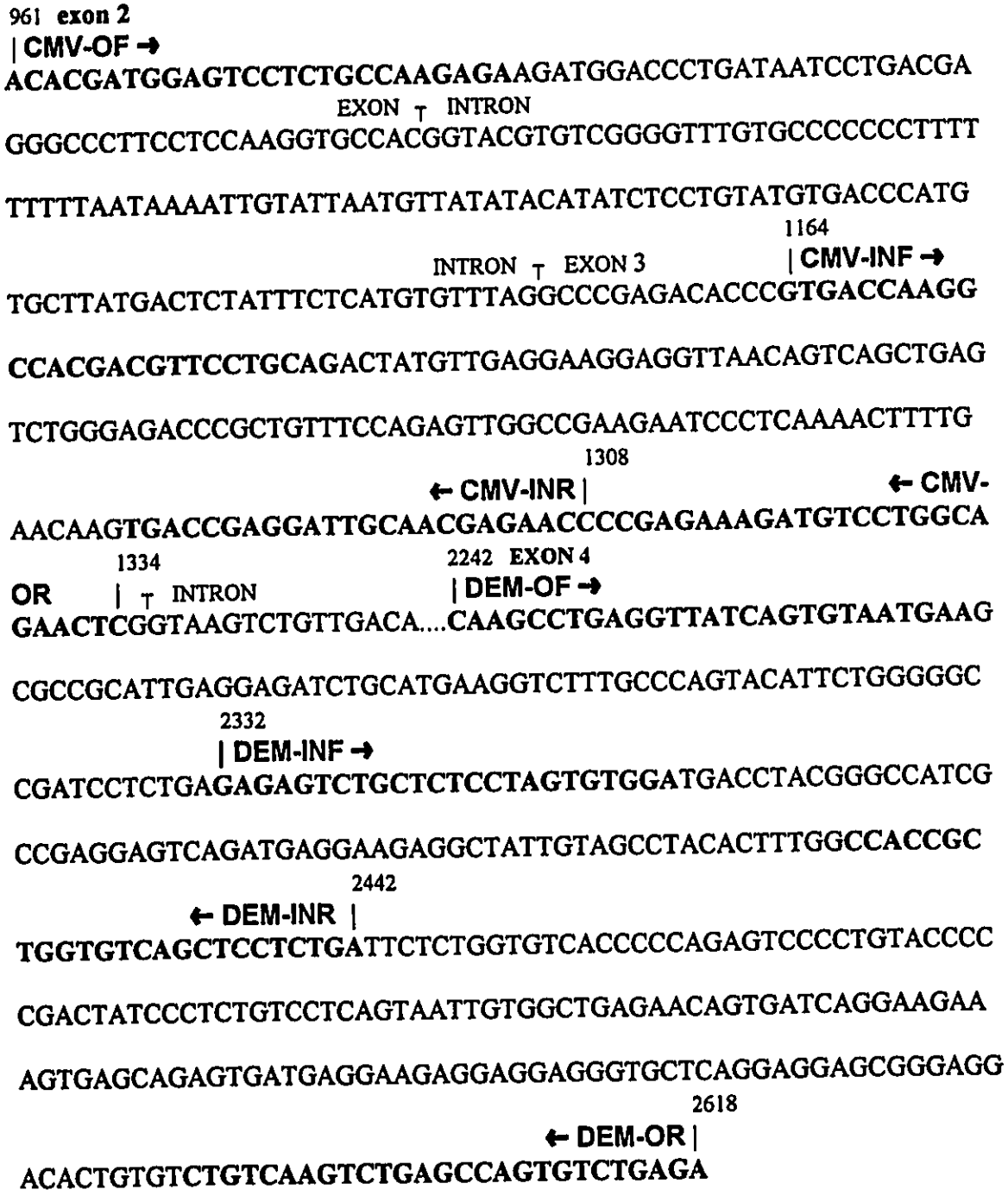
After optimization was complete, the following protocol was adapted for use in amplification of clinical specimens. 25 µL of a PCR master mix [1.4 µ*M* each of the appropriate primer, 0.4 m*M* of each deoxynucleotide triphosphate (dNTP) for the outer primer sets, 0.1 m*M* dNTP for the inner sets, 15 m*M* Tris-HCl pH 8.3, 75 m*M* KCl, 3.75 m*M* $MgCl_2$ 1 Unit of Amplitaq DNA polymerase (Perkin Elmer Cetus)] was combined with 12.5 µL sterile, distilled water in a 0.5 mL microcentrifuge tube (Perkin Elmer), overlaid with mineral oil and placed in a 80°C heating block. 12.5 µL of a cell lysate, prepared as described above, was similarly heated to the same temperature and was added to the PCR mix containing the primers such that the final concentration of the reaction components was 0.67

TABLE 1. PRIMER SEQUENCES FOR HCMV PCR.

PRIMER NAME	PRIMER DESCRIPTION	LENGTH	POSITION*	SEQUENCE (5'-3')	PRODUCT SIZE
MIE-2A	outer forward	25 nt	961-985	ACA CGA TGG AGT CCT CTG CCA AGA G	732 bp
MIE-4B	outer reverse	25 nt	1669-1693	TAG CCC AAT ACA CTT CAT CTC CTC G	
MIE-3A	inner forward	26 nt	1163-1188	CGT GAC CAA GGC CAC GAC GTT CCT GC	172 bp
MIE-3B	inner reverse	25 nt	1311-1335	CGA GTT CTG CCA GGA CAT CTT TCT C	
CMV-OF	outer forward	26 nt	961-986	ACA CGA TGG AGT CCT CTG CCA AGA GA	374 bp
CMV-OR	outer reverse	26 nt	1309-1334	GAG TTC TGC CAG GAC ATC TTT CTC GG	
CMV-INF	inner forward	27 nt	1164-1190	GTG ACC AAG GCC ACG ACG TTC CTG CAG	145 bp
CMV-INR	inner reverse	24 nt	1285-1308	GGT TCT CGT TGC AAT CCT CGG TCA	
DEM-OF	outer forward	27 nt	2242-2268	CAA GCC TGA GGT TAT CAG TGT AAT GAA	377 bp
DEM-OR	outer reverse	27 nt	2592-2618	TCT CAG ACA CTG GCT CAG ACT TGA CAG	
DEM-INF	inner forward	24 nt	2332-2355	GAG AGT CTG CTC TCC TAG TGT GGA	111 bp
DEM-INR	inner reverse	25 nt	2418-2442	TCA GAG GAG CTG ACA CCA GCG GTG G	

*Nucleotide position in sequence of immediate early gene region as per Akrigg, A *et al* (36).

FIGURE 2. LOCATION OF PRIMER SEQUENCES. A part of the MIE sequence of HCMV AD169 is provided in the 5'-3' direction as per Akrigg *et al* (36). The location of exons and introns is indicated above the sequence. The 2 sets of nested primer sets chosen for amplification of clinical samples are listed in bold as well as their starting nucleotide position as per reference 36. The direction of primer elongation is indicated by arrows. For the reverse primers, the actual primer sequence is complementary to the sequence in bold and, by convention, is listed in table 1 in the 5'-3' direction.


961 exon 2
| CMV-OF →
ACACGATGGAGTCCTCTGCCAAGAGAAGATGGACCCTGATAATCCTGACGA
EXON ┬ INTRON
GGGCCCTTCCTCCAAGGTGCCACGGTACGTGTCGGGGTTTGTGCCCCCCCTTTT
TTTTTAATAAAATTGTATTAATGTTATATACATATCTCCTGTATGTGACCCATG
1164
INTRON ┬ EXON 3
| CMV-INF →
TGCTTATGACTCTATTTCTCATGTGTTTAGGCCCGAGACACCCGTGACCAAGG
CCACGACGTTCCTGCAGACTATGTTGAGGAAGGAGGTTAACAGTCAGCTGAG
TCTGGGAGACCCGCTGTTTCCAGAGTTGGCCGAAGAATCCCTCAAAACTTTTG
1308
← CMV-INR |
← CMV-
AACAAGTGACCGAGGATTGCAACGAGAACCCCGAGAAAGATGTCCTGGCA
1334
2242 EXON 4
OR | ┬ INTRON
| DEM-OF →
GAACTCGGTAAGTCTGTTGACA....CAAGCCTGAGGTTATCAGTGTAATGAAG
CGCCGCATTGAGGAGATCTGCATGAAGGTCTTTGCCCAGTACATTCTGGGGGC
2332
| DEM-INF →
CGATCCTCTGAGAGAGTCTGCTCTCCTAGTGTGGATGACCTACGGGCCATCG
CCGAGGAGTCAGATGAGGAAGAGGCTATTGTAGCCTACACTTTGGCCACCGC
2442
← DEM-INR |
TGGTGTCAGCTCCTCTGATTCTCTGGTGTCACCCCCAGAGTCCCCTGTACCCC
CGACTATCCCTCTGTCCTCAGTAATTGTGGCTGAGAACAGTGATCAGGAAGAA
AGTGAGCAGAGTGATGAGGAAGAGGAGGAGGGTGCTCAGGAGGAGCGGGAGG
2618
← DEM-OR |
ACACTGTGTCTGTCAAGTCTGAGCCAGTGTCTGAGA

μM each primer, 0.2 mM each dNTP (0.05 mM for nested amplification), 10 mM Tris, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% each of Tween-20 and Nonidet P-40 and 1 U Amplitaq with a final reaction volume of 50 µL. After the first amplification, 2 µL of the reaction was transferred into a fresh tube containing the same reaction components and the volume was brought up to 50 µL by the addition of 10.5 µL of lysis buffer. The thermoprofile was the same for all amplifications: 94°C for 30 seconds for template denaturation followed by 55°C for 45 seconds to allow the primers to anneal to the target sequence with a subsequent incubation at 72°C for 60 seconds to allow for DNA synthesis. This profile was carried out in an automated thermocycler (Perkin Elmer).

The number of cycles carried out differed for each primer set. For the outer primer sets, 30 cycles were carried out for CMV while 20 cycles were performed for DEM. For the CMV and DEM inner primer sets, it was 25 and 30 cycles, respectively.

Amplification of clinical samples was carried out in duplicate for each primer set. In other words, 4 amplifications were carried out per sample, 2 with the CMV primer set and 2 with the DEM primer set.

II.6 CRITERIA FOR POSITIVITY

In order for a given clinical blood sample to be considered HCMV DNA positive, all 4 out of 4 amplifications performed had to be positive. If 3 out of 4 amplifications were positive, a "+/-" or indeterminate designation was used and if 0, 1 or 2 out of 4 amplifications were positive, a "-" or negative was assigned to the sample.

II.7 DETECTION OF PCR AMPLIFICATION PRODUCTS

8 μL of the amplification product were mixed with 2 μL of 5 X loading buffer (0.1% each of bromophenol blue and xylene cyanol FF, 20% w/v sucrose) and loaded onto a 2% agarose (Bio-Rad) horizontal slab gel. Electrophoresis was carried out at 5 volts per centimetre (lengthwise) for two hours in a 1 X TBE (90 m*M* Tris-HCl, 90 m*M* boric acid, 2 m*M* EDTA, pH 8.0) buffer. Both the gel and running buffer contained 0.5 mg/mL of ethidium bromide (EtBr) allowing for direct visualization of DNA bands when the gel was exposed to short wave ultraviolet illumination. A molecular weight ladder was also run on each gel consisting of DNA fragments of known size allowing for estimation and confirmation of product size. The 1 kb ladder (Gibco BRL) was initially used but was later replaced with the 100 bp ladder (Gibco BRL) whose fragment sizes are multiples of 100 base pairs.

Gels were photographed using Polaroid Type 57 high speed film.

II.8 OPTIMIZATION EXPERIMENTS

For all of the optimization experiments, dilutions of the HCMV infected fibroblasts described above were used. The amount of virus being amplified was expressed in one of two ways: either as the amount of 50% tissue culture infectious dose units ($TCID_{50}$) or as the number of infected fibroblasts present in the reaction.

II.8.1 ALTERATION OF THERMOPROFILE

Initially, only the MIE primer set was tested. Then, the CMV and DEM primer sets were introduced. The thermoprofile for the MIE primers was quite different from the other

two sets. In the beginning, it was 94°C for 1 minute, 52°C for 2.5 minutes and 72°C for 8 minutes (3 minutes for the nested reaction) for 25 cycles, as per Porter-Jordan *et al* (148). While no formal comparison of the different thermoprofiles were done, the conditions for amplification gradually changed out of the desire to shorten cycling time: the extension time at 72°C was reduced first to 5 minutes for the outer reaction and then finally the protocol of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds was adopted for both the outer and nested reactions.

As for the CMV and DEM primer sets, the initial thermoprofile used was the one just described. It was then changed to the thermoprofile as stated in the *Standardized Protocol for HCMV Amplification* (Section II.5).

II.8.2 ADJUSTMENT OF CYCLE NUMBER

The effects of cycle number was assessed by amplifying dilutions of cell culture virus with all three of the nested primer sets using the buffer concentrations as stated in the *Standardized Protocol for HCMV Amplification.* The following combinations were tested with the first number representing the number of cycles for the outer amplification while the second number represents the inner amplification: (1) 30 & 20 cycles; (2) 20 & 30 cycles; (3) 30 & 25; (4) 25 & 30.

II.8.3 ADJUSTMENT OF dNTP CONCENTRATION

All of the components in the reaction were as in the *Standardized Protocol for HCMV Amplification* except for the dNTP concentration. 3 different concentrations of dNTPs (200μM, 50 μM, 25 μM) were used in control amplifications of HCMV Davis infected

fibroblasts and their effects, if any, were noted on the quality of amplification in terms of the strength of the specific product band and in the amount of background generated for both the CMV and DEM primer sets. This was done for the inner primer sets only.

II.8.4 MAGNESIUM TITRATION

A magnesium titration was performed for the CMV and DEM primer pairs. Magnesium concentrations between 0.5 to 2.5 m*M* in 0.5 m*M* increments were tested using HCMV infected fibroblasts as the source of HCMV DNA. All of the buffer concentrations were as stated in the *Standardized Protocol for HCMV Amplification.*

III. RESULTS

III.1 FIRST GENERATION NESTED PCR

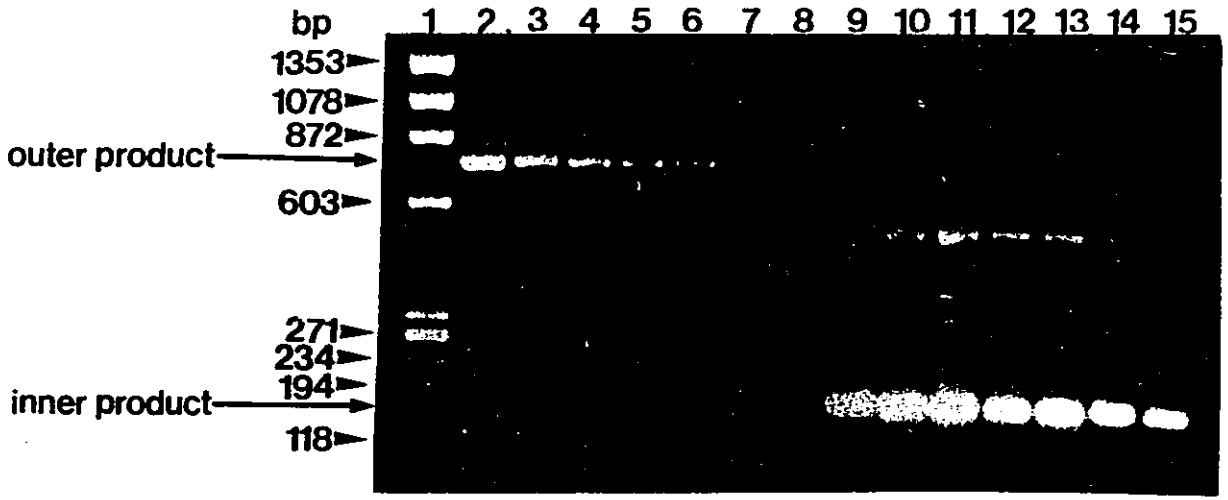
The first attempt at HCMV PCR amplification was performed with the immediate early gene region nested set of Porter-Jordan *et al* (148) and as listed in Materials and Methods. The reaction conditions that were chosen as a starting point were those for the optimized HIV PCR assays of Cassol *et al* (156) but using the thermoprofile of Porter-Jordan *et al*. That is, the outer reaction was performed at 94°C for 1 minute, 52°C for 2 minutes and 30 seconds and 72°C for 8 minutes while the inner reaction was the same except for 72°C for 3 minutes. Both amplifications were performed for 25 cycles each.

Dilutions of HCMV Davis infected fibroblasts were amplified according to this protocol and both the outer and inner reactions were run on an agarose gel. As can be seen in Figure 3, this standard protocol produced the expected bands— 732 bp for the outer reaction and 172 for the inner product.

Figure 3 also demonstrates the increase in sensitivity that can be achieved with nested PCR over a one-step PCR. By lane 8 in Figure 3, which corresponds to a $1:10^4$ dilution of the original infected fibroblast culture and containing 1.25×10^{-1} $TCID_{50}$, the product band is no longer visible on the Ethidium bromide stained agarose gel while the corresponding nested amplification product (lane 15) is still strongly visible. Dilutions past this first amplification "endpoint" still result in the clear production of the nested band (data not shown). The last dilution at which a band was seen was at the 10^{-7} dilution (1.25×10^{-4}

FIGURE 3. ONE STEP VERSUS NESTED PCR. Dilutions of DNA from HCMV Davis infected fibroblasts with a total titer of 1.6×10^5 $TCID_{50}$ were first amplified with the MIE 2A/4B and subsequently with the nested MIE 3A/3B primers. 8 μL of the amplified products were run in a 2% agarose gel and stained with EtBr. The amplification conditions used are as described in Materials & Methods, *Alteration of Thermoprofile.* The thermoprofile was 94°C for 1 minute, 52°C for 2.5 minutes and 72°C for 8 minutes (3 minutes for inner reaction). The long arrows point to the specific HCMV PCR products of the MIE 2A/4B (outer product) primer set and the MIE 3A/3B (inner product) primer set, respectively. The lane assignments, showing the dilutions used with the corresponding amount of virus expressed in $TCID_{50}$ in brackets, are as follows:

	MIE 2A/4B (outer reaction)		MIE 3A/3B (inner reaction)
Lane	1: 1kb DNA ladder (1 μg)	Lane	9: 1:100 dilution (40)
	2: 1:100 dilution (40)		10: 1:200 (20)
	3: 1:200 (20)		11: 1:400 (10)
	4: 1:400 (10)		12: 1:600 (5)
	5: 1:600 (5)		13: 1:800 (2.5)
	6: 1:800 (2.5)		14: 1:1000 (1.25)
	7: 1:1000 (1.25)		15: 1:10000 (0.125)
	8: 1:10000 (0.125)		


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1353
1078
872
outer product
603
271
234
194
inner product
118

$TCID_{50}$), while no band appeared at the $1:10^8$ dilution (data not shown). Thus, nested PCR is at least 1000 times more sensitive than a one step PCR.

III.2 INTRODUCTION OF TWO NEW PRIMER SETS

Demmler *et al* (90) in their HCMV PCR analysis indicated that in order to detect all clinical isolates, it was necessary to use more than one primer set. Thus, the CMV and DEM nested primer sets were introduced into the laboratory.

The initial reaction conditions used for these primer pairs were the same as for the MIE primer set in terms of buffer components. However, the thermoprofile was different and shorter times could be used at each temperature because of the substantially smaller product sizes (732 v 374 bp) for instance. Thus, the thermoprofile initially chosen was 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds.

III.3 OPTIMIZATION OF PCR ASSAYS

An initial concern in the optimization experiments was the desire to reduce the presence of background, high molecular weight bands that can be significant. Thus, the dNTP and magnesium concentrations and the number of cycles performed per amplification were varied in order to determine their effects on the yield of specific and non-specific amplification products.

III.3.1 ADJUSTMENT OF CYCLE NUMBER

The number of cycles performed for each amplification were varied between 20 and 30 cycles using the following combinations for the outer and inner reactions, respectively: (1) 30 and 25 cycles; (2) 25 and 30 cycles; (3) 20 and 30 cycles and (4) 30 and 20 cycles. The thermoprofile used was 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds. Five different dilutions of HCMV Davis infected fibroblasts were amplified with each of these conditions. Interestingly, when comparing the results of the three different primer sets, they performed optimally with different cycle number combinations, as shown in Figure 4. The MIE primer set performed the best with the 30 and 20 cycle combination. The CMV primer set shows the most specific product and least background with the 30 and 25 cycle combination while the DEM primer set performs the best with the 20 and 30 cycle combination. Thus, future work with the primer sets were performed with these different cycle combinations. Because the MIE primer set performed poorly compared to the other 2 primer sets and because only 2 primer sets were needed, it was dropped from use and future experiments used the CMV and DEM sets.

III.3.2 ADJUSTMENT OF dNTP CONCENTRATION

Three different dNTP concentrations, 200 μM, 50 μM, 25μM were tested in the nested reaction. The dNTP concentration for the outer reaction was kept constant at 200 μM. Figure 5 demonstrates that changing the dNTP concentration had little effect both on the amount of amplified product and background bands although a decrease is evident at 25 μM for the DEM primer set. Thus, the intermediate dNTP (50 μM) concentration was chosen for further experiments.

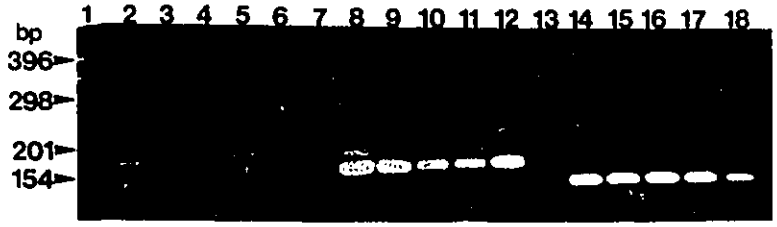
FIGURE 4. EFFECT OF CYCLE NUMBER ON AMPLIFICATION EFFICIENCY FOR THE MIE, CMV AND DEM PRIMER SETS. Dilutions of DNA from HCMV Davis infected fibroblasts with a total titer of 1.6×10^5 $TCID_{50}$ were amplified with all three of the above primer sets. The conditions for amplification—except for the number of cycles performed—and thermoprofile are as described in Material & Methods, *Standardized Protocol for HCMV PCR Amplification.* The thermoprofile was 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds. Four different cycle number combinations for the outer and inner reactions were tested as shown in each part of the figure: (A) 30 & 25 cycles; (B) 25 & 30; (C) 20 & 30 and (D) 30 & 20. 8 µL of the nested amplification products were run in a 2% agarose gel and stained with EtBr. The lane assignments are as follows:

Lane 1: 1kb DNA ladder (1µg)
2:●1:2.0 x 10^6 dilution, MIE primers
3: 1:4.0 x 10^6, MIE primers
4: 1:8.0 x 10^6, MIE primers
5: 1:1.6 x 10^7, MIE primers
6: 1:3.2 x 10^7, MIE primers
7: HCMV negative DNA control
8: 1:2.0 x 10^6 CMV primers
9: 1:4.0 x 10^6, CMV primers

Lane 10: 1:8.0 x 10^6, CMV primers
11: 1:1.6 x 10^7, CMV primers
12: 1:3.2 x 10^7, CMV primers
13:*HCMV negative DNA control
14: 1:2.0 x 10^6, DEM primers
15: 1:4.0 x 10^6, DEM primers
16: 1:8.0 x 10^6, DEM primers
17: 1:1.6 x 10^7, DEM primers
18: 1:3.2 x 10^7, DEM primers

● Lane 2 is omitted in C because this dilution was not tested.
* Lane 8 amplified product was mistakenly reloaded in lane 13 in B

A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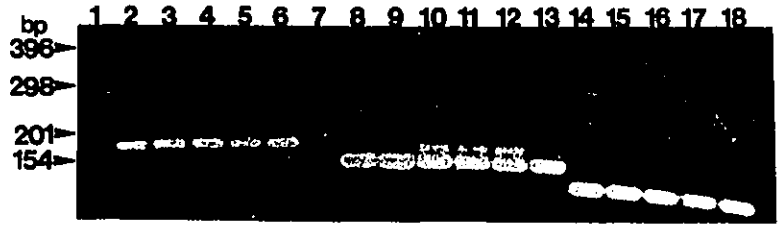
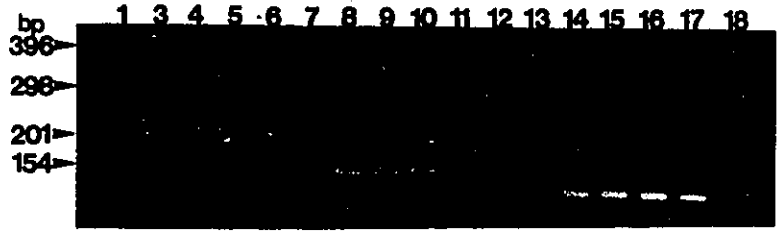
bp
396
298
201
154

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
bp
396
298
201
154

C


1 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
bp
396
298
201
154

D

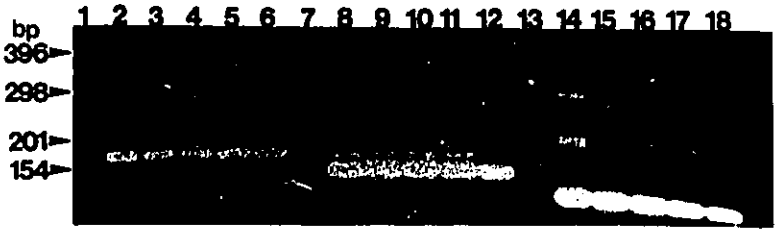
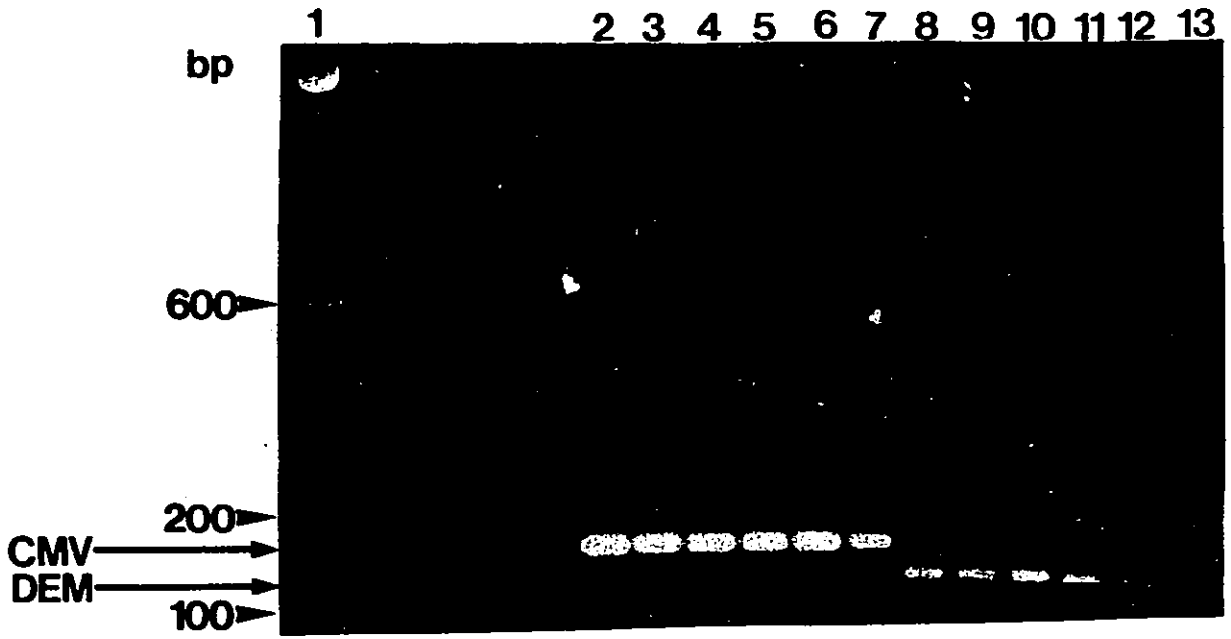

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
bp
396
298
201
154

FIGURE 5. EFFECT OF DNTP CONCENTRATION ON AMPLIFICATION EFFICIENCY OF THE NESTED REACTION FOR BOTH CMV AND DEM PRIMER SETS. A vial of HCMV Davis infected fibroblasts from ATCC was counted, the cells lysed in lysis buffer and the DNA diluted to the equivalent of either 1 or 10^{-2} infected cell (i.c.) per reaction. The dNTP concentration of the outer reaction remained at 200 μM while concentrations of 200, 50 and 25 μM were tested in the nested reaction. 8µL of the amplified products were run in a 2% agarose gel and stained with EtBr. The thermoprofile and number of cycles of amplification performed are as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* The long arrows point to the CMV and DEM nested PCR products, respectively. The lane assignments are as follows:

	CMV PRIMER SET		DEM PRIMER SET
Lane	1: 100bp DNA ladder (1µg)	Lane	8: 1 i.c., 200 μM
	2: 1 i.c., 200 μM		9: 10^{-2} i.c., 200 μM
	3: 10^{-2} i.c., 200 μM		10: 1 i.c., 50 μM
	4: 1 i.c., 50 μM		11: 10^{-2} i.c., 50 μM
	5: 10^{-2} i.c., 50 μM		12: 1 i.c., 25 μM
	6: 1 i.c., 25 μM		13: 10^{-2} i.c., 25 μM
	7: 10^{-2} i.c., 25μM		


1
2 3 4 5 6 7 8 9 10 11 12 13
bp
600
200
CMV
DEM
100

III.3.3 ADJUSTMENT OF Mg CONCENTRATION

The magnesium ion concentration also influences the efficiency of the PCR. Magnesium concentrations in the range of 0.5-2.5 m*M* for the inner reaction were tested, again using dilutions of HCMV Davis infected fibroblasts as a source of viral DNA. The outer amplification magnesium ion concentration remained at 2.5 m*M*. Figure 6 shows that the effect of Mg in this assay in this concentration range was minimal, except at the lower end of the range (lanes 16 & 17) where a sharp drop in product is qualitatively apparent. Thus, the 2.5 m*M* concentration remained unchanged.

III.4 SENSITIVITY OF HCMV PCR

Having optimized the assays, it was then necessary to establish how much of the virus the PCR assays can detect. This was done in two ways. First, by using dilutions of $TCID_{50}$ units, where HCMV Davis infected fibroblasts from ATCC containing a known amount of $TCID_{50}$ were serially diluted. The dilutions were then amplified and the lowest $TCID_{50}$ giving a signal with the MIE primer set was used as an endpoint. In the second approach, the number of fibroblasts in such a vial were counted, lysed and the DNA diluted with these dilutions being amplified as in the first approach but instead using the CMV and DEM primer sets.

Both primer sets show the same sensitivity levels as shown in Figures 7 and 8. In terms of $TCID_{50}$ units, a $1:10^7$ dilution of the original stock of 1.6×10^6 $TCID_{50}$ could be reproducibly amplified with the MIE primer set corresponding to 1.25×10^{-4} $TCID_{50}$ of

FIGURE 6. EFFECT OF MG ION CONCENTRATION ON AMPLIFICATION EFFICIENCY OF THE NESTED REACTION FOR BOTH CMV AND DEM PRIMER SETS. A vial of HCMV Davis infected fibroblasts from ATCC was counted, the cells lysed in lysis buffer and the DNA diluted to the equivalent of either 1 or 10^{-2} infected cell (i.c.) per reaction. The Mg ion concentration of the outer reaction remained at 2.5 m*M* while concentrations of 2.0, 1.5, 1.0 and 0.5 m*M* were tested in the nested reaction. All reactions were amplified in duplicate. 8 µL of the amplified products were run in a 2% agarose gel and stained with EtBr. The thermoprofile and number of cycles of amplification performed are as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* The top photograph shows the nested CMV primer amplification products while the bottom photograph shows the nested DEM primer products with the long arrows in each photograph pointing to the specific HCMV amplification band. The lane assignments (for both gels) are as follows:

Lane 1: 100 bp DNA ladder (1µg)
2: 1 i.c., 2.0 m*M*
3: " " " "
4: 10^{-2} i.c., 2.0 m*M*
5: " " " " "
6: 1 i.c., 1.5 m*M*
7: " " " "
8: 10^{-2} i.c., 1.5 m*M*

Lane 9: 10^{-2} i.c., 1.5 m*M*
10: 1 i.c., 1.0 m*M*
11: " " " "
12: 10^{-2} i.c., 1.0 m*M*
13: " " " " "
14: 1 i.c., 0.5 m*M*
15: " " " "
16: 10^{-2} i.c., 0.5 m*M*
17: " " " " "

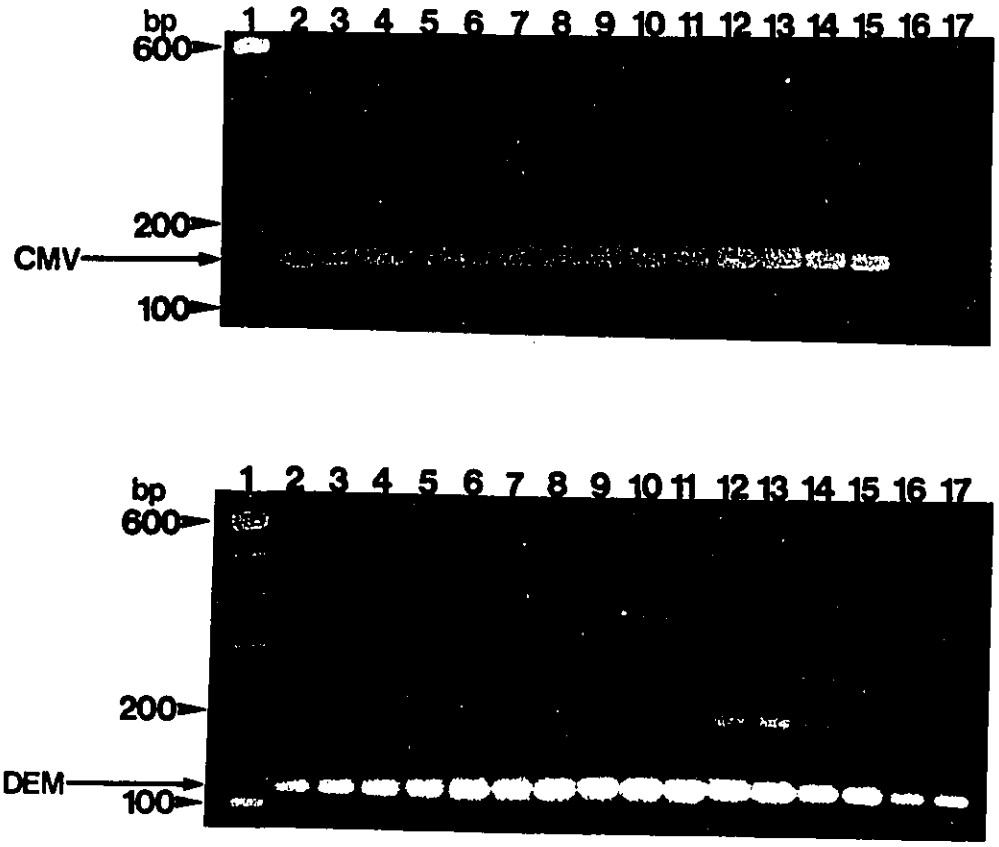
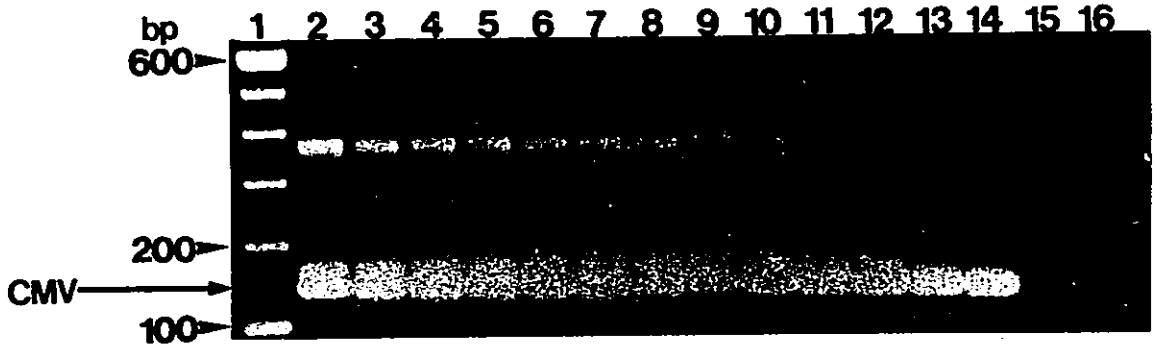

bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
600
200
CMV
100
bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
600
200
DEM
100

FIGURE 7. SENSITIVITY OF THE HCMV PCR ASSAYS. A vial of HCMV Davis infected fibroblasts from ATCC was counted, the cells lysed in lysis buffer and the DNA diluted into HCMV negative DNA. The amount of HCMV DNA present is expressed as the number of infected fibroblasts (i.c.) per reaction (range: 1 to 3000). Nested PCR was performed using both the CMV (top photograph) and DEM (bottom photograph) nested primer sets as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* 8 µL of the nested reaction products were run in a 2% agarose gel and stained with EtBr. The specific HCMV PCR products are indicated by the long arrows. The lane assignments (for both gels) are as follows:

Lane 1: 100 bp DNA ladder (1 µg)
2: 3000 i.c.
3: 2000 i.c.
4: 1000 i.c.
5: 500 i.c.
6: 250 i.c.
7: 125 i.c.
8: 62 i.c.

Lane 9: 31 i.c.
10: 15 i.c.
11: 7 i.c.
12: 5 i.c.
13: 1 i.c.
14: 1 i.c.
15: HCMV negative DNA control
16: Reagent (no DNA) control


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
600
200
CMV
100

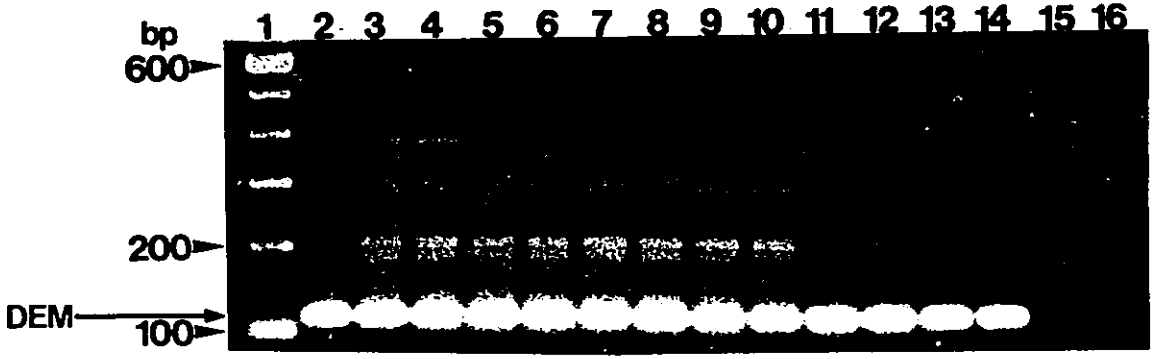
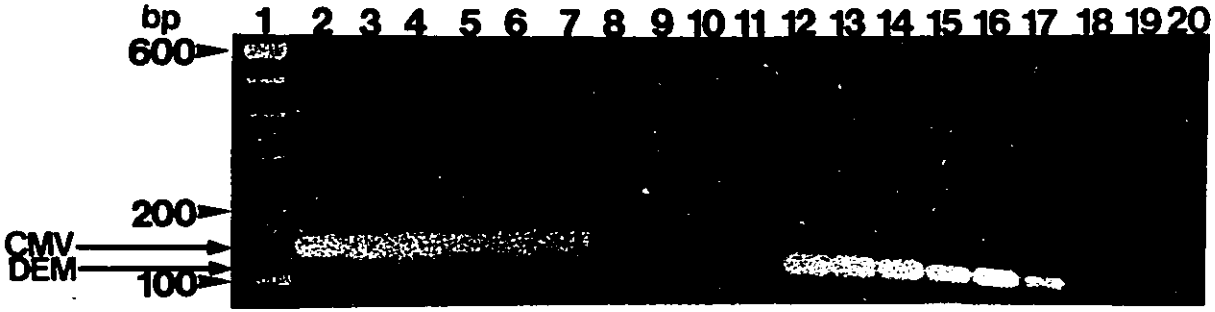

bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
600
200
DEM
100

FIGURE 8. SENSITIVITY OF THE HCMV PCR ASSAYS (CONTINUED). A vial of HCMV Davis infected fibroblasts from ATCC was counted, the cells lysed in lysis buffer and the DNA diluted in buffer to the equivalent of 10^{-1} to 10^{-4} of an HCMV infected cell. Nested PCR was performed using both the CMV and DEM nested primer sets in duplicate on these dilutions as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* 8 µL of the nested reaction products were run in a 2% agarose gel and stained with EtBr. The specific HCMV PCR products are indicated by the long arrows. The lane assignments are as follows:

	CMV PRIMER SET		DEM PRIMER SET
Lane	1: 100 bp DNA ladder (1 µg)	Lane	11: Reagent control
	2: 10^{-1} i.c.		12: 10^{-1} i.c.
	3: " " "		13: " " "
	4: 10^{-2} i.c.		14: 10^{-2} i.c.
	5: " " "		15: " " "
	6: 10^{-3} i.c.		16: 10^{-3} i.c.
	7: " " "		17: " " "
	8: 10^{-4} i.c.		18: 10^{-4} i.c.
	9: " " "		19: " " "
	10: Reagent (no DNA) control		20: Reagent control


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
600
200
CMV
DEM
100

HCMV. In terms of the number of HCMV infected cells that could be amplified, 1 infected cell per reaction produced a strong signal (lanes 13 & 14, Figure 7). Even a 1000 fold dilution of DNA from a single infected fibroblast resulted in reproducible and strong amplification. (lanes 16 & 17, Figure 8), while products were no longer visible at the 10000 fold dilution (lanes 18 & 19, Figure 8). Also of note is that while the specific HCMV PCR product band remains very strong even at the 10^{-3} dilution, the level of background bands significantly drops and below 1 infected cell per reaction disappears altogether.

III.5 SPECIFICITY OF HCMV PCR

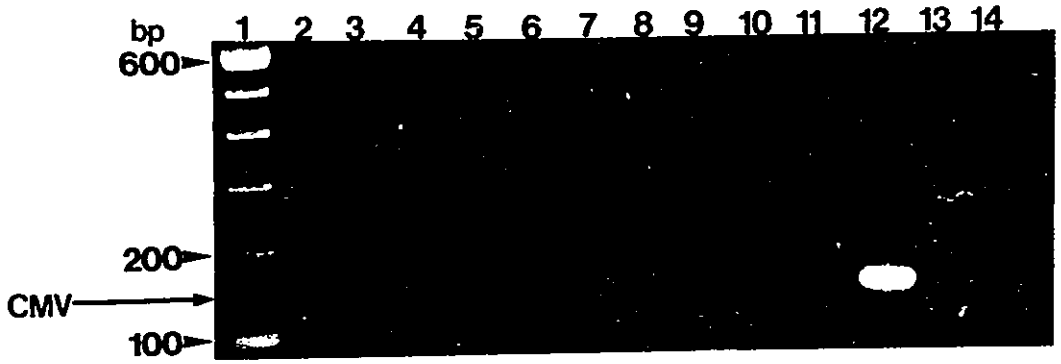
A useful PCR must amplify only the specific target and not other partially homologous cellular or viral sequences. Thus, the optimized PCR conditions were used to amplify DNA from cells infected with other human Herpesviruses. Figure 9 clearly shows that there is no specific product from the DNA of any of these viruses when using the HCMV specific primers although several high molecular weight non-specific products are apparent with the DEM primer set (Figure 9).

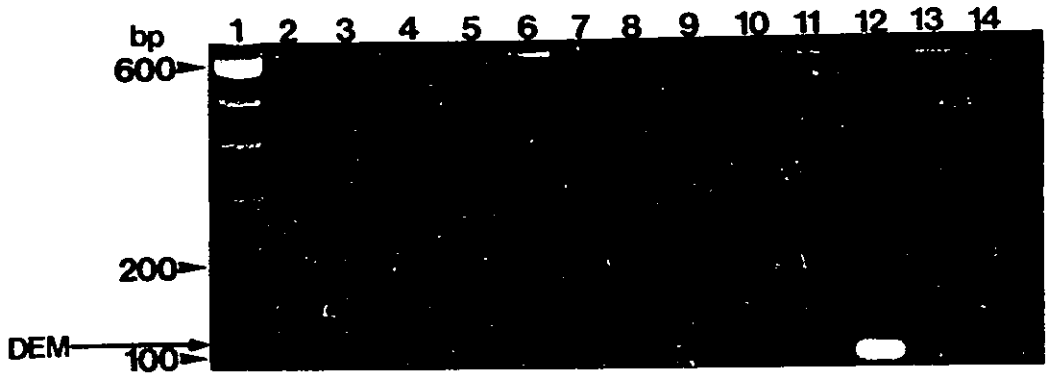
III.6 CLINICAL SAMPLES

The optimized assays were then used in a study to determine if they would detect HCMV in clinical specimens. PBMCs from normal donors, HIV infected asymptomatic individuals and AIDS patients were lysed and HCMV PCR amplified. The results for each group will be described separately.

FIGURE 9. SPECIFICITY OF THE HCMV PCR ASSAYS. Amplification of Herpesvirus DNA with the optimized HCMV nested PCR. PCR was performed in duplicate using both the CMV (top photograph) and DEM (bottom photograph) nested primer sets on DNA from cells infected with each of the known human herpesviruses. 8 µL of the nested reaction products were run in a 2% agarose gel and stained with EtBr. The specific HCMV PCR products are indicated by the long arrows. The lane assignments (for both gels) are as follows:

Lane 1: 100 bp DNA ladder (1 µg)
2: HSV-1 DNA
3: " " " "
4: HSV-2 DNA
5: " " " "
6: EBV DNA
7: " " "
8: VZV DNA
9: " " " "
10: HHV-6 DNA
11: " " " "
12: HCMV DNA (10^{-3} i.c.)
13: HCMV negative DNA control
14: Reagent (no DNA) control


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
600
200
CMV
100


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
600
200
DEM
100

III.6.1 HIV SERONEGATIVE BLOOD DONORS

PBMC DNA from Red Cross blood donors was amplified to determine if HCMV was detectable. Out of 14 normal donors (Table 2), none were consistently positive for HCMV although there were occasional amplifications but again, using the criteria that amplification must be reproducible in duplicate using both nested primer sets, they did not meet the criteria for positivity. ND9, for instance, was positive for the DEM primer set while negative for the CMV primer set. ND34 was positive 2 out of 4 amplifications. 8 out of the 14 normal donors were completely negative even though three out of 8 were HCMV seropositive. In addition, sporadic amplifications were seen in 2 individuals (ND9, ND19) who were tested to be HCMV seronegative. Representative amplifications are shown in Figure 10.

III.6.2 HIV POSITIVE INDIVIDUALS

The next group tested were the HIV positive individuals who had not progressed to AIDS, that is, they were CDC stage III. A total of 50 samples from 5 individuals were tested for HCMV. The results, as shown in Table 3 and Figure 11, demonstrate that the majority of these individuals do not harbour reproducible HCMV DNA—by PCR—in their peripheral blood mononuclear cells. Samples from two individuals (4004, 12501) were totally negative for HCMV DNA while the other three (1301, 15101, 19101) had sporadic but not consistent positive PCR results.

III.6.3 INDIVIDUALS WITH AIDS

Finally, 35 samples from 9 different individuals with AIDS (CDC Stage IV) were amplified. As shown in Table 4 with a representative example of amplification in Figure 12, HCMV DNA could be consistently detected in 8 out of 9 individuals (except for GALA) in

TABLE 2. HCMV PCR RESULTS FROM AMPLIFICATION OF HIV-1 SERONEGATIVE DONOR PBMC DNA.

DONOR NUMBER	HCMV SEROSTATUS	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
ND4	+	- -	- -	-
ND9	-	- -	+ +	-
ND10	+	- -	- -	-
ND13	+	+ -	+ +	+/-
ND18	-	- -	- -	-
ND19	-	- -	+ -	-
ND21	-	- -	- -	-
ND25	-	- -	- -	-
ND29	+	- -	- -	-
ND32	-	- -	- -	-
ND34	+	- +	+ -	-
ND35	+	- -	- +	-
ND37	+	- -	- +	-
ND38	-	- -	- -	-

*less than 2 reactions positive scored as "-"
3 out of 4 reactions positive scored as "+/-"
4 out of 4 reactions positive scored as "+"

FIGURE 10. REPRESENTATIVE EXAMPLE OF HCMV PCR AMPLIFICATION OF PBMC DNA FROM HIV-1 SERONEGATIVE BLOOD DONORS. Optimized PCR was performed in duplicate using both the CMV (top photograph) and DEM (bottom photograph) nested primer sets as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* 8 µL of the nested reaction products were run in a 2% agarose gel and stained with EtBr. The specific HCMV PCR products are indicated by the long arrows. The lane assignments are as follows:

Lane 1: 100 bp DNA ladder (1 µg)
2: ND25
3: " " "
4: ND29
5: " " "
6: ND32
7: " " "
8: ND34
9: ND 34
10: ND35
11: " " "
12: ND37
13: " " "
14: ND38
15: " " "
16: HCMV DNA (10^{-3} i.c. DNA)
17: Reagent (no DNA) control

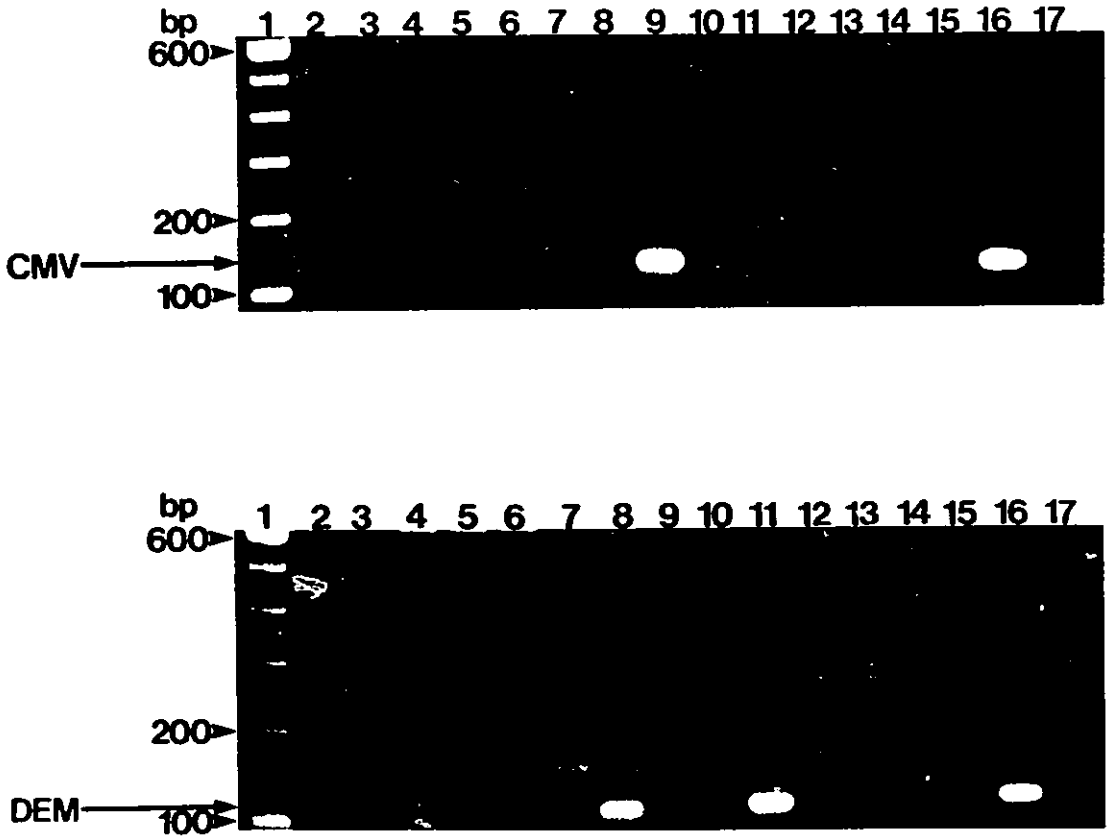

bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
600
200
CMV
100
bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
600
200
DEM
100

TABLE 3. HCMV PCR ANALYSIS OF PBMC SAMPLES FROM HIV-1 INFECTED CDC STAGE III INDIVIDUALS

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
1301	28/8/85	- -	- +	-
	4/12/85	- -	- -	-
	20/3/86	- -	- -	-
	18/6/86	- -	- -	-
	21/10/86	- -	- -	-
	20/4/87	- -	- -	-
	31/7/87	- +	+ +	+/-
	13/10/87	- -	- -	-
	12/1/88	- -	- -	-
	12/4/88	- -	-	-
	21/7/88	- -	- -	-
	24/10/88	- -	- -	-
	1/5/89	- -	- -	-
	24/1/90	- -	- -	-
	26/4/90	- -	- -	-
	8/8/90	- -	- -	-

TABLE 3 (CONTINUED)

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
13101 (continued)	11/2/91	+ -	- +	-
4004	11/9/87	- -	- -	-
	24/2/87	- -	- -	-
	12/5/87	- -	- -	-
	4/3/88	- -	- -	-
	13/6/88	- -	- -	-
	5/12/88	- -	- -	-
	29/5/89	- -	- -	-
	27/11/89	- -	- -	-
12501	11/12/86	- -	- -	-
	13/3/87	- -	- -	-
	19/1/88	- -	- -	-
	3/1/89	- -	- -	-
	23/3/89	- -	- -	-
	20/6/89	- -	- -	-
	11/10/89	- -	- -	-

TABLE 3 (CONTINUED)

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
15101	9/2/87	- +	- -	-
	4/5/87	- +	- -	-
	2/11/87	- +	- -	-
	4/8/87	- -	- -	-
	4/8/88	- +	- -	-
	24/10/88	+ -	- -	-
	4/4/89	- +	- -	-
	21/6/89	+ -	- -	-
	20/9/89	+ -	- -	-
	2/1/90	- -	- -	-
19101	5/5/87	- +	- -	-
	19/11/87	- -	- -	-
	17/2/88	- -	- -	-
	23/11/88	- -	- -	-
	24/4/88	- -	- -	-
	31/5/89	- -	- -	-

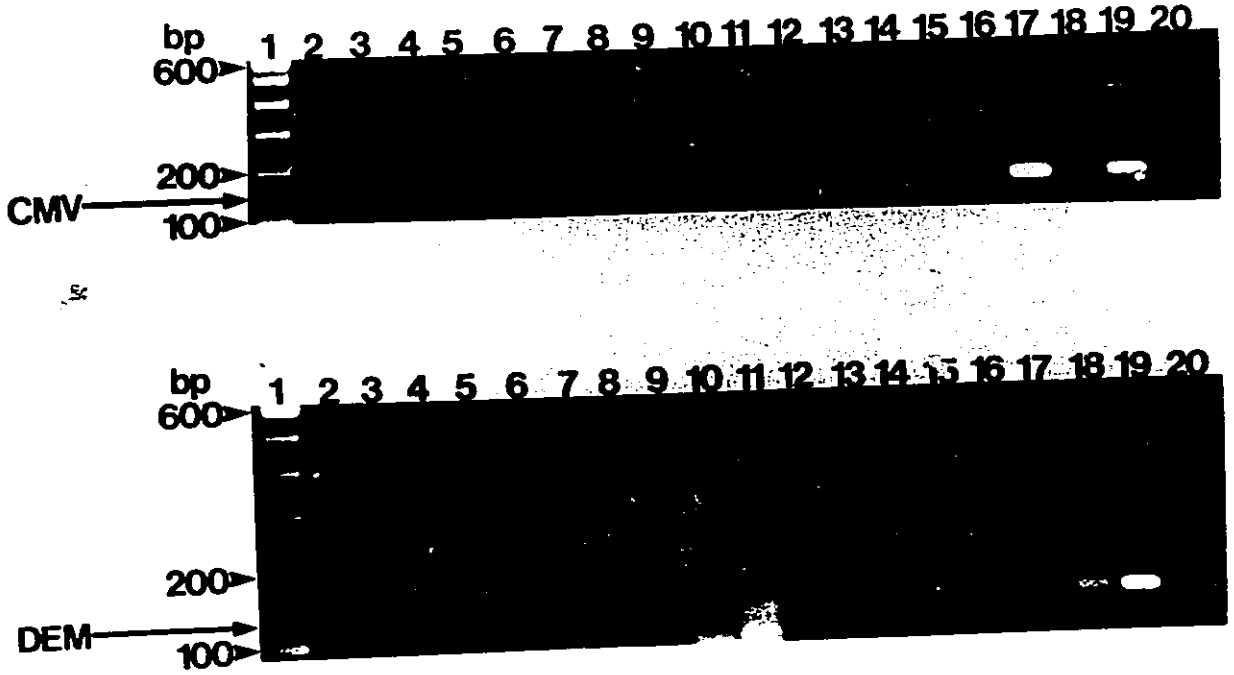
TABLE 3 (CONTINUED)

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
19101 (continued)	5/9/89	- -	- -	-
	5/12/89	- -	- -	-

*less than 2 reactions positive scored as "-"
3 out of 4 reactions positive scored as "+/-"
4 out of 4 reactions positive scored as "+"

FIGURE 11. REPRESENTATIVE EXAMPLE OF HCMV PCR AMPLIFICATION OF PBMC DNA FROM AN HIV-1 SEROPOSITIVE CDC STAGE III INDIVIDUAL (#1301, Table 3). Optimized PCR was performed in duplicate using both the CMV (top photograph) and DEM (bottom photograph) nested primer sets as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* 8µL of the nested reaction prodcuts were run in a 2% agarose gel and stained with EtBr. The specific HCMV PCR products are indicated by the long arrows. The lane assignments, indicating the date that the blood sample was taken, are as follows:

Lane 1: 100 bp DNA ladder (1µg)
2: 12/1/88
3: 12/4/88
4: " " "
5: 21/7/88
6: " " "
7: 24/10/88
8: " " "
9: 1/5/89
10: " " "
Lane 11: 24/1/90
12: " " "
13: 26/4/90
14: " " "
15: 8/8/90
16: " " "
17: 11/2/91
18: " " "
19: Positive control (10^{-3} i.c. DNA)
20: Reagent (no DNA) control


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
600
200
CMV
100
bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
600
200
DEM
100

at least one of their samples. Two patterns could be seen: (1) those that were consistently and repeatedly positive over time (141, 184) and (2) those that were less consistently positive (e.g. 56). Turning to the individuals with only one or two leukocyte samples available (the last 5 individuals of Table 4), only one (GALA) did not have HCMV amplified from the PBMC sample. Of the 4 individuals with more than 2 serial samples available, 2 of the 4 developed clinically diagnosed HCMV retinitis. In patient 141, HCMV retinitis was diagnosed in May 1991, while HCMV retinitis was diagnosed in September 1990 in patient 184. Thus, PCR detected HCMV in PBMCs 14 and 7 months, respectively, before the development of clinically detectable retinitis. Patient 141 died in October 1991, and 184 in February 1991. In the 2 others (11 and 56), no clinically overt CMV mediated disease was apparent but 11 died in December 1990.

TABLE 4. HCMV PCR ANALYSIS OF PBMC SAMPLES FROM INDIVIDUALS WITH AIDS (CDC STAGE IV).

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
11.5	9/1/90	+ +	+ -	+/-
11.6	8/3/90	+ +	+ +	+
11.7	5/4/90	+ +	+ +	+
11.8	2/5/90	- +	+ +	+/-
11.9	28/6/90	+ +	+ +	+
56.1	20/12/89	+ +	+ -	+/-
56.3	7/3/90	- -	- -	-
56.4	10/4/90	+ +	+ +	+
56.5	2/5/90	+ -	+ -	-
56.6	29/5/90	- -	+	+/-
56.7	4/7/90	+ -	+ -	+/-
56.8	1/8/90	+ -	+ +	+/-
56.9	4/10/90	- -	- -	-
56.10	1/11/90	- -	- -	-
56.11	5/12/90	+ -	+ +	+/-
56.12	12/12/90	+ +	+	+

TABLE 4 (CONTINUED)

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
141.6	4/1/90	- -	- -	-
141.7	2/2/90	- +	+ +	+/-
141.8	22/2/90	- +	+ +	+/-
141.9	22/3/90	+ +	+ +	+
141.10	19/4/90	+ +	- -	+/-
141.11	16/5/90	+ +	+ +	+
141.12	3/7/90	+ +	+ +	+
141.13	1/8/90	+ +	+ +	+
141.14	3/10/90	+ +	+ +	+
184.4	2/2/90	+ +	+ +	+
184.5	10/4/90	+ +	+ +	+
184.6	23/5/90	+ +	+ +	+
184.8	31/10/90	+ +	+ +	+
LORE	19/10/90	+ +	+ +	+
	9/11/90	+ +	+ +	+
GALA	2/11/90	+ -	- -	-

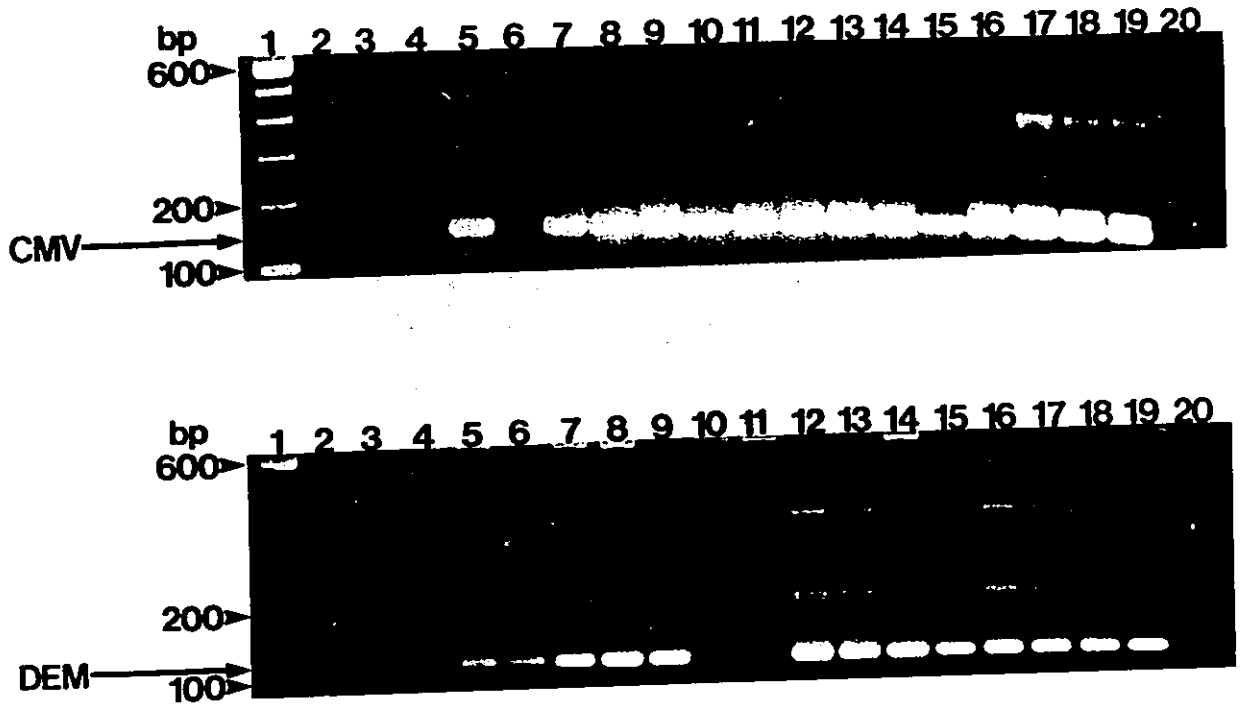
TABLE 4 (CONTINUED)

SAMPLE NUMBER	SAMPLE DATE	CMV PRIMER SET	DEM PRIMER SET	HCMV PCR RESULT*
GELV	1/11/90	+ +	+ +	+
DEGI	9/11/90	+ +	+ +	+
REST	23/11/90	+ +	+ +	+

* less than 2 reactions positive scored as "- "
3 out of 4 reactions positive scored as "+/- "
4 out of 4 reactions positive scored as "+ "

FIGURE 12. REPRESENTATIVE EXAMPLE OF HCMV PCR AMPLIFICATION OF PBMC DNA FROM AN HIV-1 SEROPOSITIVE STAGE IV (AIDS) INDIVIDUAL (#141, Table 4). Optimized PCR was performed in duplicate using both the CMV (top photograph) and DEM (bottom photograph) nested primer sets as described in Materials & Methods, *Standardized Protocol for HCMV PCR Amplification.* 8μL of the nested reaction products were run in a 2% agarose gel and stained with EtBr. The specific HCMV PCR products are indicated by the long arrows. The lane assignments, indicating the date that the PBMC sample was taken, are as follows:

Lane 1: 100 bp DNA ladder (1 μg)
2: 141.6, 4/1/90
3: " " " "
4: 141.7, 2/2/90
5: " " " "
6: 141.8, 22/2/90
7: " " " "
8: 141.9, 22/3/90
9: " " " "
10: 141.10, 19/4/90
11: 141.10, 19/4/90
12: 141.11, 16/5/90
13: " " " "
14: 141.12, 3/7/90
15: " " " "
16: 141.13, 1/8/90
17: " " " "
18: 141.14, 3/10/90
19: " " " "
20: Reagent (no DNA) control


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
600
200
CMV
100
bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
600
200
DEM
100

IV. DISCUSSION

The polymerase chain reaction is becoming an increasingly important technique used both in basic molecular biological science and in the detection of microbial pathogens in humans.

PCR for the detection of HCMV represents an attractive alternative to other detection systems in terms of specificity, sensitivity and assay time. HCMV PCR can be performed in one day. Culture of the virus, although being the gold standard, can take up to 6 weeks because of the notoriously slow growth of the virus. A high degree of technical skill and expense is also required for the successful culture of the virus. Enhancements in culture techniques, such as the shell vial assay, can decrease the culture time but again technical skill as well as ensuring the availability of fresh, viable culture cells can be disadvantageous. Serological testing, while useful for determining who has or hasn't been exposed to the virus is not very useful in differentiating a latent from active infection, particularly in individuals who are immunodeficient or in populations who have a high proportion of seropositivity. Immunohistochemical staining of immediate early or early HCMV antigens in blood cells is another alternative, but non-specific staining, particularly with immunoperoxidase, must be carefully controlled for and again a high degree of technical skill and fresh top-quality reagents are prerequisites for valid results. Finally, DNA hybridization techniques suffer the disadvantages of requiring biohazardous, expensive and short shelf-life radioisotopes along with possible cloning vector homology problems with the sample DNA. PCR, on the other hand, can be made to be highly specific, sensitive without the need for isotopes especially if

nested PCR is performed and can be rapid—all desirable qualities when attempting to detect HCMV or any other genetic sequence in a clinical sample.

As a starting point for the use of HCMV PCR, the primers of Porter-Jordan *et al* (148) were synthesized and used in combination with the optimized buffer conditions of the HIV-1 PCR assay of Cassol *et al* (156). Nested PCR was chosen because it offers an increase in sensitivity over one step PCR without the need for resorting to a secondary detection system like isotopic or fluorescent labeled probes which are often used after a one step PCR. The increase in sensitivity achieved through the use of nested PCR is demonstrable in Figure 3 where the nested PCR reaction product continues to be seen even after the amplification product of the first reaction is no longer visible. In fact, it was approximately 1000 times more sensitive than the one step PCR.

In addition to sensitivity and specificity, a good PCR assay, especially if it is going to be used as a routine test on a large-scale basis, has to be reproducible and as rapid as possible. The primers initially selected from Porter-Jordan *et al* (148, the MIE primers in Table 1) as well as the thermoprofile that they used did not satisfy these goals. The outer product size was much too large (732 bp) for a nested reaction product of 172 bp. Furthermore, the thermoprofile that they suggested was unnecessarily long with an annealing time of 2.5 minutes and an extension time of 8 and 3 minutes, for the outer and inner reactions, respectively. For a 30 cycle reaction, this thermoprofile could take 3 to 5 hours or longer. Not only is this much too long to make the assay convenient for use in a working day, it is also unnecessary. Given that the inner product is only 172 bp in size, it would be

advantageous to minimize the outer product size to, for example, 300 to 400 bp. This design would allow for the maximization of sensitivity and reproducibility since the efficiency of amplification of a product is inversely proportional to the size of the product while minimizing the time it takes to amplify the products since shorter cycling times could be used to amplify the smaller target sequence.

To this end, two alternate nested primer sets were chosen with outer product sizes about half the size of the MIE 2A/4B product size. With these new primer sets, the time for amplification was approximately 1 to 2 hours depending on the number of cycles performed which is a considerable time savings over the Porter-Jordan *et al* protocol.

The choice of primers is important in any PCR assay but it is especially important when dealing with HCMV because of the homologies both with human sequences (127) and the other herpesviruses, especially HHV-6 (151). Therefore, to have a truly specific HCMV PCR assay, these homologous genomic areas must be avoided when designing primers. The lack of sequence data from HCMV strains found *in vivo* also makes primer choices more limited because it is necessary to have primers that amplify the majority of HCMV strains found *in vivo* . Sequence data from wild-type virus would allow for the assessment of which genomic areas are well conserved between different strains and which are more variable. Currently, primer selection is based primarily on the sequence of the HCMV laboratory strain AD169 and to a lesser extent the Towne strain.

The target chosen for amplification was the major immediate early gene region of HCMV. Given its extreme importance in regulating the gene expression of both the virus (1)

and to some extent cellular gene expression (39, 40), sequence conservation can be expected to be quite high in this area and so primers designed to amplify the IE gene region would be expected to recognize most of the wild type strains. The use of two distinct primer sets increases the likelihood that a given clinical isolate is amplifiable. This presumption of sequence conservation of the IE gene region is somewhat confirmed by Demmler *et al* (90) who found that their IE PCR primers amplified all of 46 clinical isolates that they examined, but that two primer sets were necessary in order to detect all of the isolates from urine. The immediate early gene region, therefore, would seem to be a near-perfect target for amplification.

The initial experiments involving amplification of the HCMV infected fibroblasts demonstrated that the 2 PCR assays were already working quite well. Work then focused on optimizing the reaction conditions to try and reduce what is widely referred to as "non-specific" amplification products which are generally extra bands that are seen on gel electrophoresis of PCR products, while still maintaining sensitivity. This optimization involved altering the number of cycles of amplification performed and the dNTP and magnesium concentration of the nested reaction. The results showed that the reaction is quite robust. The dNTP and magnesium concentration did not have a dramatic effect on the amount of non-specific product generated nor on the amount of the expected PCR product at the concentrations tested as shown by the respective titrations in Figures 5 and 6. What did have a more pronounced effect, however, was the number of cycles of amplification performed. The best cycle combination differed for each of the primer sets with the CMV primer set having an optimal combination of 30 and 25 cycles for the outer and inner

reactions, respectively, and the DEM set's combination of 20 and 30 cycles seemed to be optimal for these primers. The amount of these non-specific products that are generated is thought to depend on several factors including the concentration of buffer components, the number of cycles of amplification performed as well as on the amount of initial target DNA present. Clearly in these HCMV PCR assays, the amount of target DNA present in the reaction is of major importance. As the amount of target DNA decreased, so too did the amount of background while the specific PCR product still remained very strong (Figures 7 and 8). However, because the specific product band is always the most predominant band with the background or non-specific bands being minor species, for the sake of simplicity and savings of time, both primer sets could be used with the same conditions.

As always, the demonstration of specificity and sensitivity is obligatory in any PCR assay. The HCMV primers used here showed absolutely no cross reactivity with any of the other known human herpesviruses, except for the generation of a minor amount of non-specific product of higher molecular weight than the HCMV specific product seen with the DEM primer set (Figure 9). This lack of cross-reactivity further confirms that the immediate early gene region of HCMV is a good choice for the detection of the virus by PCR.

The sensitivity was assessed by amplifying dilutions of HCMV Davis infected fibroblasts with the amount of virus present in the reaction expressed in $TCID_{50}$ units or as the number of infected fibroblasts in the reaction. Both the CMV and DEM primer sets showed the same sensitivity levels, amplifying up to a 10^{-3} dilution of one infected cell. While this dilution may appear to be unusual at first, consider that in a productively infected cell where new virus is being produced, multiple copies of the HCMV genome are being

generated so conceivably one productively infected cell could contain hundreds and perhaps thousands of genomic DNA copies. In one particular study, for instance (157), a relationship was determined between $TCID_{50}$ and the amount of viral genomes present as determined by quantitative PCR—$TCID_{50}$ underestimated the number of genomes present 1000 fold. Furthermore, Benyesh-Melnick *et al* (158) showed that the number of HCMV particles/plaque forming unit (pfu) for strain AD169 was between 160 to 1050. Relating the $TCID_{50}$ data to the infected cell dilution data, it can be estimated that one productively infected fibroblast is equivalent to 0.4 $TCID_{50}$ or that 1 $TCID_{50}$ is equivalent to 2.5 productively infected fibroblasts. It is reasonable, therefore, to be able to detect HCMV DNA in a reaction containing the DNA of a portion of a productively infected cell.

How does the sensitivity of this HCMV PCR assay compare to other published assays where their sensitivities are provided? This can be a difficult comparison to make as different papers express the assay sensitivities in different ways. The way in which the amplification products are detected, be it just by gel electrophoresis of amplified material or through the use of a radiolabelled probe, will also affect the level of sensitivity achieved. For example, Shibata *et al* (159) report being able to detect one pfu of cell free virus with the use of a radiolabelled probe. Demmler *et al* (90) estimate their limit of detection to be $10^{1.3}$ $TCID_{50}$ /0.1 mL again with the use of a radiolabelled probe and Cassol *et al* (160) detected 1 viral genome per 40000 cells in their system. Using a nested PCR reaction, Brytting *et al* (161) were able to detect one AD169 infected fibroblast. Finally, Jiwa *et al* (124) report a detection limit of about 60 copies of HCMV DNA after a one-step amplification without hybridization with a probe. As can be seen, the sensitivity of different assays can vary widely

although it would appear that our HCMV assays offer detection that appears to be at least as sensitive as anything currently published.

With the optimized PCR ready for use, the assays were next applied to lysed human peripheral blood mononuclear cells to determine if HCMV was detectable in clinical specimens. Three small groups of samples were tested with the results from each to be discussed separately.

IV.1 HCMV IN THE BLOOD OF HIV NEGATIVE BLOOD DONORS

Single blood samples from 14 HIV negative blood donors were tested for the presence of HCMV DNA. Using the strict criteria that all four amplifications (2 amplifications per primer set) must be positive in order to declare a HCMV positive result, none of the normal donors were HCMV DNA positive. However, that is not to say that the blood samples were entirely clean of HCMV DNA. As is evident in Table 2, HCMV DNA could be sporadically amplified from these normal donor samples. Even more interesting is that in two individuals who are seemingly seronegative for HCMV (ND9 in Table 2), the DEM primer set yielded a positive amplification signal. For the most part however, individuals seronegative for HCMV had no HCMV DNA detectable while those seropositive showed a limited amount of positive signals.

The finding of HCMV DNA in seronegative individuals is somewhat difficult to explain as it would be expected that those who have been exposed to the virus would mount an immune response against the virus. Possible explanations for this discrepancy include that

perhaps the individuals are actually seropositive for HCMV but the serological test yielded a false negative result or that the presence of HCMV specific PCR product in these individuals is a false positive generated by contaminating HCMV DNA. This is always a danger given the exquisite sensitivity of PCR. However, given all the precautions that are routinely taken to prevent contamination from occurring and given that the reagent controls were negative, one hesitates to dismiss these results as simply being due to carry-over contamination.

A survey of the literature on this subject indicates that this is not a novel finding. In three separate publications by two different groups (81, 82, 162), HCMV PCR was used to examine blood from normal blood donors. In the first report (81), 3 out of 25 donors had a PCR positive result from blood samples even though they were seronegative. Both the PCR and serology results were reproducible, even with repeat testing 6 months later. A Western blot analysis was weakly positive for 2 out of the three donors. A further investigation by this same group (162) confirmed their previous observations. Finally, a report in 1991 (82) claimed to have reproducibly detected HCMV DNA in 3 out of 9 seronegative subjects. What we are left with, then, is the possibility that HCMV is present in individuals who do not mount an immune response against the virus or alternatively the serological tests being used lack the sensitivity to detect all wild type strains due to antigenic heterogeneity of the virus.

The finding of HCMV DNA by PCR in HCMV seropositive individuals albeit not reproducibly, is also substantiated by the above (81, 82, 162). While the virus is generally not culturable from the blood of HCMV seropositive asymptomatic individuals with the exception

of one report in 1969 (66), the virus must be present in some form in the blood at least sporadically since a small percentage of blood from seropositive donors can transmit HCMV. However, since the assays cannot reproducibly detect the virus in the seropositive individuals tested here, this may indicate that the virus is present in quantities just at the borderline of detectability by the PCR assays such that enough of the DNA is available only sporadically in any given sample from the individual.

IV.2 HCMV IN THE BLOOD OF HIV-1 SEROPOSITIVE INDIVIDUALS

IV.2.1 ASYMPTOMATIC INDIVIDUALS

50 blood samples from 5 different individuals were tested in this group providing an opportunity to determine, over several years, whether HCMV could be detected in this group. Unfortunately, the HCMV serostatus of these individuals is not known although given that they are all homosexual men and if other studies of homosexual cohorts (63) are any indication, the majority of these individuals will be seropositive for HCMV, upwards of 90% or more.

Nevertheless, as with the HIV-1 seronegative group tested, none of the 50 samples could be called positive using the strict criteria for positivity. But again, in some samples there were sporadic positives that could be due to either contamination of the sample by exogenous HCMV DNA or that the amount of viral DNA present is at the limit of detection. Contamination is unlikely, however, because the negative control was negative and the lack of large amounts of HCMV DNA present in these individuals greatly reduces the chances that

carry-over contamination can occur. For the most part, however, HCMV was not detectable in the peripheral blood mononuclear cells of HIV-1 seropositive individuals (Table 3).

IV.2.2 INDIVIDUALS WITH AIDS

In the 35 samples from 9 individuals with AIDS, HCMV was detected in the blood in 8 of the 9 individuals reproducibly by PCR. Clearly they had more HCMV DNA in their blood than the other two groups.

These results are also confirmed in the literature (100, 101) where a progressive increase in HCMV viremia occurs as HIV progresses from the asymptomatic stages to the advanced disease stage. This makes sense in terms of the disease process. During the course of an HIV infection, the immune response diminishes especially at the end stages of AIDS when the CD4 cell count is drastically decreased. Opportunistic infections can then flourish as the result of the immunodeficiency. HCMV, like all herpesviruses, remains in the body after a primary infection and as a result of immune system collapse, is allowed to reactivate and cause disease like retinitis in individuals with AIDS.

IV.3 LINGERING QUESTIONS

The extreme sensitivity of PCR enabling it to detect the smallest quantities of viral DNA leads many to ask the important question of what clinical relevance is a positive PCR result? The mere presence of the amplified viral DNA does not necessarily mean that the virus is actively replicating or that it is the cause of any morbidity. The PCR could be detecting latent virus, for example. The results shown here, however, suggests that these

PCR assays may be differentiating an active from a latent infection. It is clear that the site of HCMV persistence within the body is still not known although it is clear that the virus can be found in the blood. In the PCR results for the blood donors and HIV seropositive individuals, these individuals did not have HCMV disease, nor did they have reproducible HCMV PCR amplifications from their blood. In individuals with AIDS, some had known HCMV mediated disease and were reproducibly HCMV PCR positive although there were some individuals who were PCR positive but did not have clinically detectable HCMV mediated disease. Therefore, the difference in PCR reproducibility suggests that there is more HCMV within the blood of AIDS patients (as compared to the blood donors and HIV seropositive, non-AIDS persons) and that the PCR is detecting HCMV involved in an active infection. The best PCR method to distinguish between latency and active replication of HCMV in blood cells would be to do "reverse transcriptase PCR." This involves isolating the RNA from the PBMCs, reverse transcribing the RNA and then performing HCMV PCR on the newly generated complementary DNA. A more appropriate primer choice in this instance would be primers directed against late structural genes which are transcribed only in active infection.

Another method to distinguish between an active and latent infection would be to do a quantitative PCR to determine the amount of virus present within a sample. There may be a threshold level of HCMV DNA present within the PBMCs above which indicates an active infection and below which indicates a latent or persistent infection. These are refinements in the PCR technique that may yield more useful information than the technique used in this study.

The data presented here would suggest that a reproducible positive PCR result may be clinically relevant and that it maybe a better and earlier predictor of oncoming HCMV disease. Although culture data are not available for the majority of the samples, of the 6 samples where culture data are available (the last 6 rows of Table 4), 5 out of the 6 were both PCR and culture positive. Given the rapidity of the HCMV PCR assay over culture, it represents an attractive alternative for the detection of the virus. In terms of predicting the onset of HCMV disease, a positive PCR result can mean that HCMV mediated disease is occurring or is about to occur but it does not seem to necessarily predict the onset of clinically overt disease. Of the individuals with AIDS who had serial blood samples taken (Table 4) two individuals with somewhat sporadic PCR positive results (11 and 56 in Table 4) had no detectable HCMV disease. However, in two others (141 and 184) who had reproducible PCR positive results, PCR for HCMV was positive 14 and 7 months, respectively, before HCMV retinitis was clinically diagnosed. The presence of HCMV in the blood, as detected by PCR, can therefore mean that HCMV has reactivated and is potentially attacking tissues. This has important implications with respect to initiating anti-HCMV therapies in order to address the issue of whether starting therapy earlier before the onset of overt disease can improve the clinical outcome. HCMV retinitis is clinically diagnosed and so significant damage to the retina can be done before the disease is even detected. The use of PCR to monitor the state of an HCMV infection may mean that sight-threatening and/or life-threatening disease can be avoided.

It also should be noted that HCMV disease, even a disseminated infection, often goes undetected while an individual is alive. Recent autopsy studies of individuals who have died

from AIDS-related illnesses (86, 105-107) demonstrate that HCMV infections are often not clinically diagnosed and so its widespread presence *in vivo* may contribute to the significant morbidity in these individuals.

Other questions that still remain to be satisfactorily answered include what blood cells are infected by HCMV although mononuclear cells are implicated here as in other published reports (69-83) and is HCMV contributing to HIV disease progression? If HCMV is a cofactor in disease progression to AIDS, than one would expect that the virus would be present in an active rather than latent form and present in sufficient quantity to have an effect on the immune system or on HIV directly. This, in turn, would suggest that HCMV should be detectable **prior** to the development of AIDS and not **due** to the development of AIDS. The fact that HCMV is easily detectable in peripheral blood quite readily in AIDS patients but not in earlier stages of the disease would argue against the idea of a cofactorial relationship. On the other hand, restricting the analysis of any such relationship to the peripheral blood may not be reflective of the entire *in vivo* situation. What if HCMV and HIV interact in a location other than the peripheral blood? From studies of peripheral blood, for example, it was believed that HIV was present in minute amounts during the asymptomatic stage of HIV infection. It has now been suggested through studies of lymph node tissue of HIV infected individuals that HIV is present in higher amounts in lymph node than in peripheral blood (163). If this is also the case for HCMV, it may strengthen the relationship between what has been observed *in vitro* in terms of the effects of HCMV on HIV transcription and replication (109-118) and what may be happening *in vivo*. These questions, while not being addressed here, perhaps can be attempted to be elucidated with the use of PCR.

IV.4 HCMV PCR: THE FUTURE

Clearly, the polymerase chain reaction is becoming an important diagnostic tool for the detection of microbial pathogens. While not licensed for use in diagnostic tests as yet, the day is coming when PCR assays will be commercially available for use in making diagnostic determinations.

PCR for HCMV will be invaluable for detecting virus, for measuring responses to anti-viral therapies and in research applications such as analyzing which cell types are infected and in measuring the emergence of resistance mutants to the drugs now available and to those in development. Nested amplification of the immediate early gene region of HCMV allows for the rapid, sensitive and easy detection of the virus. It is and will be a useful research tool to attempt to uncover the unknowns about how human cytomegalovirus persists and acts *in vivo*.

V. REFERENCES

1. Ho M., editor. 1991. Cytomegalovirus: Biology and Infection. Plenum Publishing Corp, New York.

2. Goodpasture E.W., F.B. Tablot. 1921. Concerning the nature of "protozoan-like" cells in certain illnesses of infancy. Am J Dis Child 21: 415-425.

3. Von Glahn W.C., A.M. Pappenheimer. 1925. Intranuclear inclusions in visceral disease. Am J Pathol 1: 445-465.

4. Cowdry E.W. 1934. The problem of intranuclear inclusions in virus disease. Arch Pathol 18: 525-542.

5. Wyatt J.P., J. Saxton, R.S. Lee, H. Pinkerton. 1950. Generalized cytomegalic inclusion disease. J Pediatr 36: 271-294.

6. Fetterman G.H. 1952. A new laboratory aid in the clinical diagnosis of inclusion disease in infancy. Am J Clin Pathol 22: 424-425.

7. Minder W.H. 1953. Die Aetiologie der Cytomegalic infantum. Schweiz Med Woehensehr 83: 1180-1182.

8. Enders J.F., T.H. Weller, F.C. Robbins. 1949. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109: 85-87.

9. Smith M.G. 1956. Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease. Proc Soc Exp Biol Med 92: 424-430.

10. Rowe W.P., J.W. Hartley, S. Waterman, H.C. Turner, R.J. Huebner. 1956. Cytopathogenic agent resembling human salivary gland virus recovered from tissue culture of human adenoids. Proc Soc Exp Biol Med 92: 418-424.

11. Weller T.H., J.C. Macauley, J.M. Craig, P. Wirth. 1957. Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. Proc Soc Exp Biol Med 94: 4-12.

12. Chee M.A., A.T. Bankier, S. Beck, R. Bohni, C.M. Brown, R. Cerny, T. Hornell, C.A. Hutchison III, T. Kouzarides, J.A. Martignetti, E. Preddie, S.C. Satchwell, P. Tomlinson, K.M. Weston, B.G. Barrell. 1990. An analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol 154: 125-169.

13. Fields B.N., D.M. Knine, editors-in-chief. Fields Virology, vol2. 2nd edition 1990. Chapters 64, 69, 70. Raven Press, New York.

14. Mach M., T. Stamminger, G. Jahn. 1989. Human cytomegalovirus: Recent aspects from molecular biology. J Gen Virol 70: 3117-3146.

15. Lehner R., H. Meyer, M. Mach. 1989. Identification and characterization of a human cytomegalovirus gene coding for a membrane protein that is conserved among human herpesviruses. J Virol 63: 3792-3800.

16. Chang C.P., D.H. Vesole, J. Nelson, M.B.A. Oldstone, M.F. Stinski. 1989. Identification and expression of a human cytomegalovirus early glycoprotein. J Virol 63: 3330-3337.

17. Liu Y-N C., B. Kari, R.C. Gehrz. 1988. Human immune responses to major human cytomegalovirus glycoprotein complexes. J Virol 62: 1066-1070.

18. Kari B., N. Lussenhop, R. Goertz, M. Wabuke-Bunoti, R. Radeke, R. Gehrz. 1986. Characterization of monoclonal antibody reactive to several biochemically distinct human cytomegalovirus glycoprotein complexes. J Virol 60: 345-352.

19. Kari B., R. Gehrz. 1992. A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. J Virol 66: 1761-1764.

20. Grundy J.E., J.A. McKeating, P.D. Griffiths. 1987. Cytomegalovirus strain AD169 binds β_2 microglobulin *in vitro* after release from cells. J Gen Virol 68: 777-784.

21. Grundy J.E., J.A. McKeating, P.J. Ward, A.R. Sanderson, P.D. Griffiths. 1987. β_2 microglobulin enhances the infectivity of cytomegalovirus and when bound to the virus enables Class I HLA molecules to be used as a virus receptor. J Gen Virol 68: 793-803.

22. Adlish J.D., R.S. Lahijani, S.C. St. Jeor. 1990. Identification of a putative cell receptor for human cytomegalovirus. Virology 176: 337-345.

23. Taylor H.P., N.R. Cooper. 1989. Human cytomegalovirus binding to fibroblasts is receptor mediated. J Virol 63: 3991-3998.

24. Nowlin D.M., N.R. Cooper, T. Compton. 1991. Expression of a human cytomegalovirus receptor correlates with infectibility of cells. J Virol 65: 3114-3121.

25. Keay S., T.C. Merigan, L. Rasmussen. 1989. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proc Natl Acad Sci USA 86: 10100-10103.

26. Keay S., B. Baldwin. 1991. Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. J Virol 65: 5124-5128.

27. Severi B., M.P. Landini, E. Govoni. 1988. Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases. Arch Virol 98: 51-64.

28. McKeating J.A., P.D. Griffiths, R.A. Weiss. 1990. HIV susceptibility conferred to human fibroblasts by cytomegalovirus-induced Fc receptor. Nature 343: 659-661.

29. Grundy J.E., H.M. Ayles, J.A. McKeating, R.G. Butcher, P.D. Griffiths, L.W. Poulter. 1988. Enhancement of Class I HLA antigen expression by cytomegalovirus: Role in amplification of virus infection. J Med Virol 25: 483-495.

30. Sokol D.M., G.J. Demmler, G.J. Buffone. 1992. Rapid epidemiologic analysis of cytomegalovirus by using polymerase chain reaction amplification of the L-S junction region. J Clin Micro 30: 839-844.

31. Stenberg R.M., D.R. Thomsen, M.F. Stinski. 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. J Virol 49: 190-199.

32. Kouzarides T., A.T. Bankier, S.C. Satchwell, E. Preddy, B.G. Barrell. 1988. An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. Virology 165: 151-164.

33. Colberg-Poley A.M., L.D. Santomenna, P.P. Harlow, P.A. Benfield, D.J. Tenney. 1992. Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. J Virol 66: 95-105.

34. Wathen M.W., M.F. Stinski. 1982. Temporal patterns of human cytomegalovirus transcription: Mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J Virol 41: 462-477.

35. Stinski M.F., D.R. Thomsen, R.M. Stenberg, L.C. Goldstein. 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J Virol 46: 1-14.

36. Akrigg A., G.W.G. Wilkinson, J.D. Oram. 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res 2: 107-121.

37. Michelson-Fiske S., F. Horodniceanu, J.-C. Guillon. 1977. Immediate early antigens in human cytomegalovirus infected cells. Nature 270: 615-617.

38. Stenberg R.M., J. Fortney, S.W. Barlow, B.P. Magrane, J.A. Nelson, P. Ghazal. 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J Virol 64: 1556-1565.

39. Dudding L., S. Haskill, B.D. Clark, P.E. Auron, S. Sporn, E.-S. Huang. 1989. Cytomegalovirus infection stimulates expression of monocyte-associated mediator genes. J Immunol 143: 3343-3352.

40. Iwamoto G.K., M.M. Monick, B.D. Clark, P.E. Auron, M.F. Stinski, G.W. Hunninghake. 1990. Modulation of Interleukin-1 beta chain gene expression by the immediate early genes of human cytomegalovirus. J Clin Invest 85: 1853-1857.

41. Pizzorno M.C., P. O'Hare, L. Sha, R.L. Lafemina, G.S. Hayward. 1988. *trans*-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J Virol 62: 1167-1179.

42. Tevethia M.J., D.J. Spector, K.M. Leisure, M.F. Stinski. 1987. Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology 161: 276-285.

43. Davis M.G., S.C. Kenney, J Kamine, J.S. Pagano, E.-S. Huang. 1987. Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. Proc Natl Acad Sci USA 84: 8642-8646.

44. Stasiak P.C., E.S. Mocarski. 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. J Virol 66: 1050-1058.

45. Depto A.S., R.M. Stenberg. 1989. Regulated expression of the human cytomegalovirus pp65 gene: Octamer sequence in the promoter is required for activation by viral gene products. J Virol 63: 1232-1238.

46. Geballe A.P., F.S. Leach, E.S. Mocarski. 1986. Regulation of cytomegalovirus late gene expression: γ genes are controlled by posttranscriptional events. J Virol 57: 864-874.

47. Pereira L., S. Stagno, M. Hoffman, J.E. Volanakis. 1983. Cytomegalovirus-infected cell polypeptides immune-precipitated by sera from children with congenital and perinatal infections. Infect Immun 39: 100-108.

48. Landini M.P., M.C. Re, G. Mirolo, B. Baldassarri, M. La Placa. 1985. Human immune response to cytomegalovirus structural polypeptides studied by immunoblotting. J Med Virol 17: 303-311.

49. Zaia J.A., S.J. Forman, Y.-P. Ting, E. Vanderwal-Urbina, K.G. Blume. 1986. Polypeptide-specific antibody response to human cytomegalovirus after infection in bone marrow transplant recipients. J Infec Dis 153: 780-787.

50. Pande H., K. Campo, B. Tanamachi, J.A. Zaia. 1991. Human cytomegalovirus strain Towne pp28 gene: Sequence comparison to pp28 of HCMV AD169 and stable expression in chinese hamster ovary cells. Virology 184: 762-767.

51. Landini M.P., A. Ripalti, K. Sra, P. Pouletty. 1991. Human cytomegalovirus structural proteins: immune reaction against pp150 synthetic peptides. J Clin Micro 29:1868-1872.

52. Rasmussen L., J. Mullenax, M. Nelson, T.C. Merigan. 1985. Human cytomegalovirus polypeptides stimulate neutralizing antibody *in vivo.* Virology 145: 186-190.

53. Rasmussen L., C. Matkin, R. Spaete, C. Pachl, T.C. Merigan. 1991. Antibody response to human cytomegalovirus glycoproteins gB and gH after natural infection in humans. J Infec Dis 164: 835-842.

54. Quinnan G.V., N. Kirmani, A.H. Rook, J.F. Manischewitz, L. Jackson, G. Moreschi, G.W. Santos, R. Saral, W.H. Burns. 1982. Cytotoxic T cells in cytomegalovirus infection: HLA restricted T lymphocyte and non T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus in bone marrow transplant recipients. NEJM 307: 7-13.

55. Borysiewicz L.K., J.K. Hickling, S. Graham, J. Sinclair, M.P. Cranage, G.L. Smith, J.G. Patrick Sissons. 1988. Human cytomegalovirus-specific cytotoxic T cells: Relative frequency of stage-specific CTL recognizing the 72-kD immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. J Exp Med 168: 919-931.

56. Borysiewicz L.K., S. Graham, J.K. Hickling, P.D. Mason, J.G. Patrick Sissons. 1988. Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. Eur J Immunol 18: 269-275.

57. Riddell S.R., M. Rabin, A.P. Geballe, W.J. Britt, P.D. Greenberg. 1991. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. J Immunol 146: 2795-2804.

58. Hirsch M.S., D. Felsenstein. 1984. Cytomegalovirus-induced immunosuppression. Ann NY Acad Sci 437: 8-15.

59. Schrier R.D., M.B.A. Oldstone. 1986. Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus-specific human leukocyte antigen-restricted cytotoxic T-lymphocyte activity. J Virol 59:127-131.

60. Schrier R.D., G.P.A. Rice, M.B.A. Oldstone. 1986. Suppression of natural killer cell activity and T cell proliferation by fresh isolates of human cytomegalovirus. J Infec Dis 153: 1094-1091.

61. Ho M. 1990. Epidemiology of cytomegalovirus infection. Rev Infec Dis 12 (Suppl 7): S701-S710.

62. Collier A.C., J.D. Meyers, L. Corey, V.L. Murphy, P.L. Roberts, H. Hunter Handsfield. 1987. Cytomegalovirus infection in homosexual men: Relationship to sexual practices, antibody to human immunodeficiency virus, and cell-mediated immunity. Am J Med 82: 593-601.

63. Drew W.L., L. Mintz, R.C. Miner, M. Sands, B. Ketterer. 1981. Prevalence of cytomegalovirus infection in homosexual men. J Infec Dis 143: 188-192.

64. Pass R.F., C. Hutto, R. Ricks, G.A. Cloud. 1986. Increased rate of cytomegalovirus infection among parents of children attending day-care centers. NEJM 314: 1414-1418.

65. Chou S. 1990. Newer methods for diagnosis of cytomegalovirus infection. Rev Infec Dis 12 (Suppl 7): S727-S736.

66. Diosi P., E. Moldovan, N. Tomescu. 1969. Latent cytomegalovirus infection in blood donors. BMJ 4: 660-662.

67. Webster A. 1991. Cytomegalovirus as a possible cofactor in HIV disease progression. JAIDS 4 (Suppl 1): S47-S52.

68. Fiala M., J.E. Payne, T.V. Berne, T.C. Moore, W. Henle, J.Z. Montgomerie, S.N. Chatterjee, L.B. Guze. 1975. Epidemiology of cytomegalovirus infection after transplantation and immunosuppression. J Infec Dis 132: 421-433.

69. Saltzman R.L., M.R. Quirk, M. Colin Jordan. 1988. Disseminated cytomegalovirus infection: Molecular analysis of virus and leukocyte interactions in viremia. J Clin Invest 81: 75-81.

70. Dankner W.M., J. Allen McCutchan, D.D. Richman, K. Hirata, S.A. Spector. 1990. Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. J Infec Dis 161: 31-36.

71. Rinaldo C.R., P.H. Black, M.S. Hirsch. 1977. Interaction of cytomegalovirus with leukocytes from patients with mononucleosis due to cytomegalovirus. J Infec Dis 136: 667-678.

72. Howell C.L., M.J. Miller, W.J. Martin. 1979. Comparison of rates of virus isolation from leukocyte populations separated from blood by conventional and Ficoll-Paque/ macrodex methods. J Clin Micro 10: 533-537.

73. The T.H., W. van der Bij, A.P. van den Berg, M. van der Giessen, J. Weits, H.G. Sprenger, W.J. van Son. 1990. Cytomegalovirus antigenemia. Rev Infec Dis 12 (Suppl 7): S737-S744.

74. Garnett H.M. 1982. Isolation of human cytomegalovirus from peripheral blood T cells of renal transplant patients. J Lab Clin Med 99: 92-97.

75. Tocci M.J., S.C. St. Jeor. 1979. Persistence and replication of the human cytomegalovirus genome in lymphoblastoid cells of B and T origin. Virology 96: 664-668.

76. Tocci M.J., S.C. St. Jeor. 1979. Susceptibility of lymphoblastoid cells to infection with human cytomegalovirus. Infec Immun 23: 418-423.

77. Braun R.W., H.C. Reiser. 1986. Replication of human cytomegalovirus in human peripheral blood T cells. J Virol 60: 29-36.

78. Einhorn L., A. Öst. 1984. Cytomegalovirus infection of human blood cells. J Infect Dis 149: 207-214.

79. Rice G.P.A., R.D. Schrier, M.B.A. Oldstone. 1984. Cytomegalovirus infects human lymphocytes and monocytes: Virus expression is restricted to immediate-early gene products. Proc Natl Acad Sci USA 81: 6134-6138.

80. Schrier R.D., J.A. Nelson, M.B.A. Oldstone. 1985. Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. Science 230: 1048-1051.

81. Stanier P, D.L. Taylor, A.D. Kitchen, N. Wales, Y. Tryhorn, A. Stanley Tyms. 1989. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. BMJ 299: 897-898.

82. Taylor-Wiedeman J., J.G. P. Sissons, L.K. Borysiewicz, J.H. Sinclair. 1991. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. J Gen Virol 72: 2059-2064.

83. Ibanez C.E., R. Schrier, P. Ghazal, C. Wiley, J.A. Nelson. 1991. Human cytomegalovirus productively infects primary differentiated macrophages. J Virol 65: 6581-6588.

84. Wiley C.A., J.A. Nelson. 1988. Role of Human Immunodeficiency Virus and cytomegalovirus in AIDS encephalitis. Am J Pathol 133: 73- 81.

85. Nelson J.A., C. Reynolds-Kohler, M.B.A. Oldstone, C.A. Wiley. 1988. HIV and HCMV coinfect brain cells in patients with AIDS. Virology 165: 286-290.

86. Myerson D., R.C. Hackman, J.A. Nelson, D.C. Ward, J.K. McDougall. 1984. Widespread presence of histologically occult cytomegalovirus. Hum Pathol 15: 430-439.

87. Drew L.W. 1988. Cytomegalovirus infection in patients with AIDS. J Infec Dis 158: 449-456.

88. Ho M. 1981. The lymphocyte in infections with Epstein-Barr virus and cytomegalovirus. J Infec Dis 143: 857-862.

89. Stagno S., R.F. Pass, M.E. Dworsky, R.E. Henderson, E.G. Moore, P.D. Walton, C.A. Alford. 1982. Congenital cytomegalovirus infection: The relative importance of primary and recurrent infection. NEJM 306: 945-949.

90. Demmler G.J., G.J. Buffone, C.M. Schimbor, R.A. May. 1988. Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. J Infec Dis 158: 1177-1184.

91. Alford C.A., S. Stagno, R.F. Pass, W.J. Britt. 1990. Congenital and perinatal cytomegalovirus infection. Rev Infec Dis 12 (Suppl 7): S745-S753.

92. Rubin R.H. 1990 Impact of cytomegalovirus infection on organ transplant recipients. Rev Infec Dis 12 (Suppl 7): S754-S766.

93. Chou S. 1986 Acquisition of donor strains of CMV by renal-transplant recipients. NEJM 314: 1418-1423.

94. Grundy J.E., S.F. Liu, M. Super, N.J. Berry, P. Sweny, O.N. Fernando, J Moorhead, P.D. Griffiths. 1988. Symptomatic cytomegalovirus infection in seropositive kidney recipients: reinfection with donor virus rather than reactivation of recipient virus. Lancet ii: 132-135.

95. Grundy J.E., M. Super, S. Lui, P. Sweny, P.D. Griffiths. 1987. The source of cytomegalovirus infection in seropositive renal allograft recipients is frequently the donor kidney. Transplant Proc 19: 2126-2129.

96. Drew W.L., M.A. Conant, R.C. Miner, E.-S. Huang, J.L. Ziegler, J.R. Groundwater, J.H. Gullett, P. Volberding, D.I. Abrams, L. Mintz. 1982. Cytomegalovirus and Kaposi's sarcoma in young homosexual men. Lancet ii: 125-127.

97. Marmor M., A.E. Friedman-Kien, S. Zolla-Pazner, R.E. Stahl, P. Rubinstein, L. Laubenstein, D.C. William, R.J. Klein, I. Spigland. 1984. Kaposi's sarcoma in homosexual men: a seroepidemiologic case-controlled study. Ann Int Med 100: 809-815.

98. Gottlieb M.S., R. Schroff, H.M. Schanker, J.D. Weisman, P.T. Fan, R.A. Wolf, A. Saxon. 1981. *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men. Evidence of a new acquired cellular immunodeficiency. NEJM 305: 1425-1431.

99. Giorgi J.V., J.L. Fahey, D.C. Smith, L.E. Hultin, H.L. Cheng, R.T. Mitsuyasu, R. Detels. 1987. Early effects of HIV on CD4 lymphocytes *in vivo.* J Immunol 138: 3725-3730.

100. Fiala M., L.A. Cone, C.-M. Chang, E.S. Mocarski. 1986. Cytomegaloviremia increases with progressive immune deficiency in patients infected with HTLV-III. AIDS Res 2: 175-181.

101. Quinnan G.V., H. Masur, A.H. Rook, G. Armstrong, W.R. Frederick, J. Epstein, J.F. Manischewitz, A.M. Macher, L. Jackson, J. Ames, H.A. Smith, M. Parker, G.R. Pearson, J. Parrillo, C. Mitchell, S.E. Straus. 1984. Herpesvirus infections in the Acquired Immune Deficiency Syndrome. JAMA 252: 72-77.

102. Schooley R.T. 1990. Cytomegalovirus in the setting of infection with Human Immunodeficiency virus. Rev Infec Dis 12 (Suppl 7): S811-S819.

103. Bloom J.N., A.G. Palestine. 1988. The diagnosis of cytomegalovirus retinitis. Ann Int Med 109: 963-969.

104. Macher A.M, C.M. Reichert, S.E. Straus, D.L. Longo, J. Parrillo, H. Clifford Lane, A.S. Fauci. 1983. Death in the AIDS patient: role of cytomegalovirus. NEJM 309: 1454.

105. Shibata D., E.C. Klatt. 1989. Analysis of Human Immunodeficiency virus and cytomegalovirus infection by polymerase chain reaction in the acquired immunodeficiency syndrome: An autopsy study. Arch Pathol Lab Med 113: 1239-1244.

106. Mohar A., J. Romo, F. Salido, J. Jessurun, S. Ponce de León, E. Reyes, P. Volkow, O. Larraza, M. A. Peredo, C. Cano, G. Gómez, J. Sepúlveda, N. Mueller. 1992. The spectrum of clinical and pathological manifestations of AIDS in a consecutive series of autopsied patients in Mexico. AIDS 6: 467-473.

107. d'Arminio Monforte A., L. Vago, A. Lazzarin, R. Boldorini, T. Bini, S. Guzzetti, S. Antinori, M. Moroni, G. Costanzi. 1992. AIDS-defining diseases in 250 HIV-infected patients; a comparative study of clinical and autopsy diagnoses. AIDS 6: 1159-1164.

108. Crumpacker C.S., M.H. Chiazzi. 1991. Overview of cytomegalovirus infections in HIV-infected patients: current therapies and future strategies. JAIDS 4 (Suppl 11): S1-S5.

109. Ho W.-Z., J. M. Harouse, R.F. Rando, E. Gönczöl, A. Srinivasan, S.A. Plotkin. 1990. Reciprocal enhancement of gene expression and viral replication between human cytomegalovirus and Human Immunodeficiency virus type 1. J Gen Virol 71: 97-103.

110. Clouse K.A., P.B. Robbins, B. Fernie, J.M. Ostrove, A.S. Fauci. 1989. Viral antigen stimulation of the production of human monokines capable of regulating HIV1 expression. J Immunol 143: 470-475.

111. Koval V., C. Clark, M. Vaishnav, S.A. Spector, D.H. Spector. 1991. Human cytomegalovirus inhibits Human Immunodeficiency virus replication in cells productively infected by both viruses. J Virol 65: 6969-6978.

112. Markovitz D.M., S. Kenney, J. Kamine, M.S. Smith, M. Davis, E.-S. Huang, C. Rosen, J.S. Pagano. 1989. Disparate effects of two herpesviruses immediate-early gene *trans*-activators on the HIV-1 LTR. Virology 173: 750-754.

113. Duclos H., E. Elfassi, S. Michelson, F. Arenzana-Seisdedos, A. Munier, J.-L. Virelizier. 1989. Cytomegalovirus infection and trans-activation of HIV-1 and HIV-2 LTRs in human astrocytoma cells. AIDS Res Hum Retro 5: 217-223

114. Tremblay M., M. Gornitsky, M.A. Wainberg. 1989. Active replication of Human Immunodeficiency virus type 1 by peripheral blood mononuclear cells following coincubation with herpes viruses. J Med Vir 29: 109-114.

115. Rando R.F., A. Srinivasan, J. Feingold, E. Gönczöl, S. Plotkin. 1990. Characterization of multiple molecular interactions between human cytomegalovirus (HCMV) and Human Immunodeficiency virus type 1 (HIV-1). Virology 176: 87-97.

116. Barry P.A., E. Pratt-Lowe, B. Matija Peterlin, P.A. Luciw. 1990. Cytomegalovirus activates transcription directed by the long terminal repeat of the Human Immunodeficiency virus type 1. J Virol 64: 2932-2940. .

117. Ghazal P., J. Young, E. Giulietti, C. DeMattei, J. Garcia, R. Gaynor, R.M. Stenberg, J.A. Nelson. 1991. A discrete *cis* element in the Human Immunodeficiency virus Long Terminal Repeat mediates synergistic *trans* activation by cytomegalovirus immediate-early proteins. J Virol 65: 6735-6742.

118. Skolnik P.R., B.R. Kosloff, M.S. Hirsch. 1988. Bidirectional interactions between Human Immunodeficiency virus type 1 and cytomegalovirus. J Infec Dis 157: 508-514.

119. Webster A., C.A. Lee, D.G. Cook, J.E. Grundy, V.C. Emery, P.B.A. Kernoff, P.D. Griffiths. 1989. Cytomegalovirus infection and progression towards AIDS in haemophiliacs with Human Immunodeficiency virus infection. Lancet ii: 63-66.

120. Lee C.A., A.N. Phillips, J. Elford, G. Janossy, P. Griffiths, P. Kernoff. 1991. Progression of HIV disease in a haemophilic cohort followed for 11 years and the effect of treatment. BMJ 303: 1093-1096.

121. Agha S.A., L.A. Mahmoud, L.C. Archard, A.M. Abd-Elaal, S. Selwyn, A.D. Mee, J.C. Coleman. 1989. Early diagnosis of cytomegalovirus infection in renal transplant and dialysis patients by DNA-DNA hybridisation assay. J Med Virol 27: 252-257.

122. Weller T.H. 1971. The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. NEJM 285: 203-213.

123. Warren W.P., K. Balcarek, R. Smith, R.F. Pass. 1992. Comparison of rapid methods of detection of cytomegalovirus in saliva with virus isolation in tissue culture. J Clin Micro 30: 786-789.

124. Jiwa N.M., G.W. Van Gemert, A.K. Raap, F.M. Van de Rijke, A. Mulder, P.F. Lens, M.M.M. Salimans, F.E. Zwaan, W. Van Dorp, M. Van der Ploeg. 1989. Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation 48: 72-76.

125. Fedorko D.P., D.M. Ilstrup, T.F. Smith. 1989. Effect of age of shell vial monolayers on detection of cytomegalovirus from urine specimens. J Clin Micro 27: 2107-2109.

126. Ambinder R.F., P. Charache, S. Staal, P. Wright, M. Forman, S. D. Hayward, G.S. Hayward. 1986. The vector homology problem in diagnostic nucleic acid hybridization of clinical specimens. J Clin Micro 24: 16-20.

127. Rüger R., G.W. Bornkamm, B. Fleckenstein. 1984. Human cytomegalovirus DNA sequences with homologies to the cellular genome. J Gen Virol 65: 1351-1364.

128. Spector S.A., D.H. Spector. 1985. The use of DNA probes in studies of human cytomegalovirus. Clin Chem 31: 1514-1520.

129. Mullis K.B. 1990. The unusual origin of the polymerase chain reaction. Sci Amer 262: 56-65.

130. Saiki R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

131. Saiki R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

132. Chien A., D.B. Edgar, J.M. Trela. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus.* J Bact 127: 1550-1557.

133. PCR protocols: A guide to methods and applications. 1990. Academic Press. Chapters 1, 16.

134. Lynch J.R., J.M. Brown. 1990. The polymerase chain reaction: current and future clinical applications. J Med Genet 27: 2-7.

135. Erlich H.A., D. Gelfand, J.J. Sninsky. 1991. Recent advances in the polymerase chain reaction. Science 252: 1643-1651.

136. Saiki R.K., T.L. Bugawan, G.T. Horn, K.B. Mullis, H.A. Erlich. 1986. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature 324: 163-166.

137. Embury S.H., S.J. Scharf, R.K. Saiki, M.A. Gholson, M. Golbus, N. Arnheim, H.A. Erlich. 1987. Rapid prenatal diagnosis of sickle cell anemia by a new method of DNA analysis. NEJM 316: 656-661.

138. Bos J.L., E.R. Fearon, S.R. Hamilton, M. Verlaan-de Vries, J.H. van Boom, A. J. van der Eb, B Vogelstein. 1987. Prevalence of *ras* gene mutations in human colorectal cancers. Nature 327: 293-297.

139. Lee M.-S., K.-S. Chang, F. Cabanillas, E.J. Freireich, J. M. Trujillo, S.A. Stass. 1987. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 237: 175-178.

140. Scharf S.J., G.T. Horn, H.A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233: 1076-1078.

141. Innis M.A., K.B. Myambo, D.H. Gelfand, M.A.D. Brow. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc Natl Acad Sci USA 85: 9436-9440.

142. Chamberlain J.S., R.A. Gibbs, J.E. Ranier, P.N. Nguyen, C.T. Caskey. 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nuc Acid Res 16: 11141-11156.

143. Ou C.-Y., S. Kwok, S. Mitchell. 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 239: 295-297.

144. Gyllensten U.B., H.A. Erlich. 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 85: 7652-7656.

145. Triglia T, M.G. Peterson, D.J. Kemp. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. Nuc Acid Res 16: 8186.

146. Silver J., V. Keerikatte. 1989. Novel use of polymerase chain reaction to amplify cellular DNA adjacent to an integrated provirus. J Virol 63: 1924-1928.

147. Mullis K.B., F.A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol 155: 335-350.

148. Porter-Jordan K., E.I. Rosenberg, J.F. Keiser, J.D. Gross, A.M. Ross, S. Nasim, C.T. Garrett. 1990. Nested polymerase chain reaction assay for the detection of cytomegalovirus overcomes false positives caused by contamination with fragmented DNA. J Med Virol 30: 85-91.

149. Kemp D.J., D.B. Smith, S.J. Foote, N. Samaras, M.G. Peterson. 1989. Colorimetric detection of specific DNA segments amplified by polymerase chain reactions. Proc Natl Acad Sci USA 86: 2423-2427.

150. Vandenvelde C., M. Verstraete, D. Van Beers. 1990. Fast multiplex polymerase chain reaction on boiled clinical samples for rapid viral diagnosis. J Virol Met 30: 215-228.

151. Neipel F., K. Ellinger, B. Fleckenstein. 1991. The unique region of the human herpesvirus 6 genome is essentially collinear with the U_L segment of human cytomegalovirus. J Gen Virol 72: 2293-2297.

152. Chandler S.H., J.K. McDougall. 1986. Comparison of restriction site polymorphisms among clinical isolates and laboratory strains of human cytomegalovirus. J Gen Virol 67: 2179-2192.

153. Chou S. 1990. Differentiation of cytomegalovirus strains by restriction analysis of DNA sequences amplified from clinical specimens. J Infec Dis 162: 738-742.

154. Olive D.M., S. Al Mufti, M. Simsek, H. Fayez, W. Al Nakib. 1989. Direct detection of human cytomegalovirus in urine specimens from renal transplant patients following polymerase chain reaction amplification. J Med Virol 29: 232-237.

155. Centers for disease control. 1986. Classification system for Human T-Lymphotropic Virus type III/Lymphadenopathy-associated virus infections. MMWR 35: 334-339.

156. Cassol S., T. Salas, M. Arella, P. Newmann, M.T. Schechter, M. O' Shaughnessy. 1991. Use of dried blood spot specimens in the detection of Human Immunodeficiency Virus type 1 by the polymerase chain reaction. 29: 667-671.

157. Fox J.C., P.D. Griffiths, V.C. Emery. 1992. Quantification of human cytomegalovirus DNA using the polymerase chain reaction. J Gen Virol 73: 2405-2408.

158. Benyesh-Melnick M., F. Probstmeyer, R. McCombs, J.P. Brunschwig, V. Vonka. 1966. Correlation between infectivity and physical virus particles in human cytomegalovirus. J Bacteriol 5: 1555-1561.

159. Shibata D., W. J. Martin, M.D. Appleman, D.M. Causey, J.M. Leedom, N. Arnheim. 1988. Detection of cytomegalovirus DNA in peripheral blood of patients infected with Human Immunodeficiency virus. J Infec Dis 158: 1185-1192.

160. Cassol S.A., M.-C. Poon, R. Pal, M.J. Naylor, J. Culver-James, T.J. Bowen, J.A. Russell, S.A. Krawetz, R.T. Pon, D.I. Hoar. 1989. Primer-mediated enzymatic amplification of cytomegalovirus (CMV) DNA. Application to the early diagnosis of CMV infection in marrow transplant recipients. J Clin Invest 83: 1109-1115.

161. Brytting M, V.-A. Sundqvist, P. Stalhandske, A. Linde, B. Wahren. 1991. Cytomegalovirus DNA detection of an immediate early protein gene with nested primer oligonucleotides. J Virol Met 32: 127-138.

162. Stanier P., A.D. Kitchen, D.L. Taylor, A.S. Tyms. 1992. Detection of human cytomegalovirus in peripheral mononuclear cells and urine samples using PCR. Mol Cell Probes 6: 51-58.

163. Pantaleo G., C. Graziosi, J.F. Demarest, L. Butini, M. Montroni, C.H. Fox, J.M. Orenstein, D.P. Kotler, A.S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362: 355-358.