EARLY DIAGNOSIS OF CMV DISEASE IN HIV-INFECTED INDIVIDUALS USING THE POLYMERASE CHAIN REACTION

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THESIS

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ABSTRACT

Human cytomegalovirus (CMV) is a herpesvirus that is responsible for significant morbidity and mortality in the setting of the acquired immunodeficiency syndrome (AIDS). The diagnosis of CMV disease in HIV-infected individuals is complicated by the presence of latent CMV DNA in a wide range of cells and tissues. Peripheral blood mononuclear cells (PBMCs) are the primary sites of viral latency in the circulation and the presence of CMV DNA in polymorphonuclear leukocytes (PMNLs) may be more indicative of active viral replication. Antiviral therapies are available, thus making timely diagnosis of significant importance to patients at risk of developing symptomatic disease.

The objective of this work was to devise a sensitive assay for the early diagnosis of CMV infection in HIV-infected individuals at highest risk of developing clinical CMV disease based on their $CD4^+$ T cell count and clinical status. A novel PCR assay was developed. Unlike previously described methodologies, this assay can be applied to detect both latent and active CMV DNA in appropriate clinical specimens. Sensitivity and specificity were established in comparison with conventional diagnostic tests, including cell culture and serology. Other herpesvirus DNA (EBV, HSV-1, HSV-2) and bacterial *(Neisseria gonorrhoeae)* DNA were used to confirm the specificity of the assay.

A sensitive, specific and semi-quantitative PCR protocol was applied to detect both latent and active CMV DNA in appropriate clinical specimens. We hypothesized that selective PCR in neutrophils could allow the specific detection of actively replicating virions. Blood samples routinely submitted for CMV culture were shown to be acceptable for CMV PCR. Of 25 patients

studied (HIV seropositive, CD4 < 100/μl), 9 had CMV DNA in their circulating neutrophils, while only one had a positive CMV blood culture. Six out of nine (including the culture positive individual) had CMV retinitis, while other 3 had a clinical illness compatible with CMV. None of the other 16 patients (with negative neutrophil CMV PCR results) had any evidence of clinical disease attributable to CMV. Follow - up testing of 6 patients with positive baseline PCR result and treated appropriately for CMV showed negative PCR results at 1 - 3 months. This PCR test may be useful in the diagnosis and follow - up of HIV - infected individuals at risk of CMV disease.

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DEDICATION

To my parents, Cleopa and Rhoda Msuya,
for being a constant source of inspiration
and unconditional love over the years.

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
CAT	Chloramphenical acetyl transferase
CC	Cell Culture
CD	Cluster of Differentiation
CDC	Centre for Disease Control
CMI	Cell Mediated Immunity
CMV	Cytomegalovirus
CPE	Cytopathic Effect
dATP	deoxy Adenosine Tri-Phosphate
dCTP	deoxy Cytosine Tri-Phosphate
dGTP	deoxy Guanidine Tri-Phosphate
dNTPs	deoxy Nucleotide Tri-Phosphates
dTTP	deoxy Thymidine Tri-Phosphate
EBV	Epstein Barr Virus
ELISA	Enzyme-Linked Immunoabsorbent Assay
HIV	Human Immunodeficiency Virus
HSV-1	Herpes Simplex Virus, Type 1
HSV-2	Herpes Simplex Virus, Type 2
IE	Immediate Early
Ig	Immunoglobulin
LA	Late Antigens
LTR	Long Terminal Repeats
MAb	Monoclonal Antibody

NF-kb	Nuclear Factor kappa b
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMNLs	Polymorphonuclear Leukocytes
SVA	Shell Vial Assay

LIST OF REAGENTS AND MATERIALS

Agarose ultra pure	Sigma
Beta-actin primers	National Biosciences
Blocking buffer™	Boehringer Mannheim
Bromophenol blue	Sigma
Ethidium Bromide	Sigma
IE primers	National Biosciences
LA primers	National Biosciences
LA probe	National Biosciences
Polymorphprep™	Nycomed, AS
TRI REAGENT™	Molecular Research Inc.
TRI REAGENT™-LS	Molecular Research Inc.

I. INTRODUCTION

Cytomegalovirus (CMV) remains a major opportunistic pathogen in patients with AIDS (Roizman et al., 1993). It is estimated that up to 90% of patients with AIDS have evidence of CMV disease at autopsy (Ho M., 1991). Twenty five percent of HIV-seropositive individuals with CD4 lymphocyte counts below 100 cells/μl have sight or life-threatening disease attributable to CMV (Roizman et al., 1993). The exact contribution of CMV to the severe wasting seen in AIDS patients is not well understood, however mounting evidence (Hirsch et al., 1984; Webster, 1992; Lawrence, 1990 and Ho M., 1991) suggests an interaction between HIV and CMV in this regard. The management of CMV infection in such immunocompromised patients is a major clinical challenge. Although antiviral drugs effective against CMV are available, therapy requires an intensive and life-long maintenance regimen. Despite the appropriate use of these drugs according to current guidelines, the mean life expectancy of AIDS patients with CMV retinitis is only 12 months (Jacobson and Mills, 1988). It is likely that the best treatment results can be achieved when early administration of the available drugs is initiated. Early diagnosis of CMV in HIV-seropositive individuals is complicated by the presence of latent CMV DNA in a wide range of tissues and the lack of sensitive diagnostic tools. The development of a new test for earlier diagnosis of active infection would thus represent an important advance, allowing for medical intervention to be initiated more promptly, to the ultimate benefit of the patient.

I.1 Herpesviridae

Cytomegalovirus is classified as a member of the family Herpesviridae, all of which posses the ability to establish persistent and latency (Ho, M. 1991; Plummer, 1973; Roizman et al., 1993; Weller, 1970 and Wildy, 1973). The virion of herpesviridae is characterized by a core containing a linear double stranded DNA varying from 120 to 230 kilobase pairs (Kbp) (Roizman et al., 1993). The icosahedral particle, enclosed in a lipid envelope, is 100 to 130 nm in diameter (Dimmock and Primrose, 1994). The envelope which buds from the nuclear membrane of the infected cell contains viral glycoprotein spikes on the surface (Roizman et al., 1993).

Herpesviruses are widely distributed in nature - most animal species have been reported to have at least one herpesvirus (Roizman et al., 1993). Seven herpesviruses have been characterized to be associated with humans, these include herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), cytomegalovirus, varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and human herpesvirus 6 and 7 - HHV/6 and HHV/7 (Roizman et al., 1993). In addition, a newly described herpesvirus may be associated with Kaposi's sarcoma in AIDS patients (Chang et al., 1994).

I.2 Cytomegalovirus

Cytomegalovirus belongs to the subfamily Betaherpesviridae distinguished by characteristics such as species specificity, slow growth *in vitro* and salivary gland tropism (Roizman et al., 1993). CMV is a ubiquitous virus with a characteristic cytopathologic effect on the host cells involving nuclear and cytoplasmic inclusions (Gentry et al., 1988). It is thought to be the oldest type of herpes virus in evolutionary terms and the best studied

member of beta herpesviruses. The different strains of human CMV have been reported to have up to 95% homology (Roizman et al., 1993).

Sixty to eighty percent of the general healthy population is estimated to have been exposed to CMV (Roizman et al., 1993). Transmission of CMV is believed to be mediated by direct or indirect person to person contact (Roizman et al., 1993). Body fluids are the common sources of the virus and these include urine, cervical and vaginal secretions, spermatic fluids, breast milk, oropharyngeal secretions and blood (Roizman et al., 1993). In the healthy population, the virus establishes latency, with reactivation of a latent virus associated with various forms of immunosuppression (Ho M., 1991). With an increase in AIDS cases, CMV infections in this group of patients has been a major health concern (Schooley R.T., 1990). The molecular mechanisms of establishment and maintenance of CMV latency and reactivation are yet to be completely elucidated.

I.2.1 Virion and Genomic Structure

The virion structure of CMV is typical of herpes virus. (Huang et al., 1973). It consists of a core containing a double stranded DNA in a 100 nm diameter icosahedral capsid, surrounded by a tegument or matrix. A lipid bilayer envelope with virally-coded glycoprotein spikes enclose these components, making the size of mature virions to vary from 150 - 200 nm (Roizman et al., 1993). Human cells infected with CMV release three major types of virus particles, which are (Severi et al, 1992):

(a) virions with characteristic herpesvirus morphology

(b) non-infectious particles and

(c) dense bodies that lack a nucleocapsid and may lack viral DNA.

CMV DNA is the largest of the herpesviruses with a genome size of 230 kilobase pairs (Kbp) (Roizman et al., 1993). The human CMV genome has a complex arrangement of unique and repeated sequences including the existence of four genome isomers. In addition, the CMV genome contains inverted repeats flanking genomic long (L) and short (S) components. There is also a short sequence present in direct orientation at both ends of the viral genome and in inverted orientation at the L - S junction (Roizman et al., 1993).

The genome of a CMV strain AD169 has been completely sequenced (Chee et al., 1990) but little is known about the function of many of the gene families. Despite the fact that analysis of individual gene functions is not well developed, the large coding capacity of CMV includes functions that are essential as well as functions that are dispensable for viral growth in cultured cells according to site - directed mutagenesis studies (Roizman et al., 1993).

I. 2.2 The Life Cycle of CMV

The events of the replication cycle of CMV are not well understood. Studies on CMV life cycles especially at the molecular level have been hindered by its slow replication rate and restricted host range of (Roizman et al., 1993). The mechanism for CMV attachment, penetration and maturation resembles that of HSV-1 (Compton et al., 1992;). The virus attaches to the cell via a receptor or receptors that seem to be present in different cell types (Roizman et al., 1993). To date, the nature of the receptor(s) is not yet known, however, several studies have suggested that cell surface proteins ranging from 30 - 92 KDa in size appear to be involved as receptors (Adlish et al., 1990; Taylor and Cooper, 1990). CMV absorption into the cell is not competitive

with other herpesviruses, suggesting the use of a distinct receptor(s) (Roizman et al, 1993).

Following attachment to the cell, a rapid penetration occurs by direct fusion of the viral envelopes with the plasma membrane as recently suggested (Crompton et al., 1992). Upon entering the cell, viral nucleocapsids migrate towards the nucleus, where viral DNA is delivered through nuclear pores. Like other herpesviruses, synthesis and assembly of DNA occurs in the nucleus hence presenting a need for transport mechanism of viral proteins synthesized in the cytoplasm to the nucleus (Roizman et al., 1993). These early events are very rapid and efficient, as indicated by the detection of earliest classes of CMV gene expression within 20 to 30 minutes after exposure of cells to virus inoculum (Roizman et al., 1993). The mechanism by which the virus initiates cellular processes is still controversial. Some investigators have suggested the binding of the virus to the cell initiates the signal transduction cascade is sufficient even in the absence of viral penetration (Albrecht at al., 1992 and Baldogh et al., 1991). This being said, the highly restricted host range of CMV seem to be determined by a post penetration block involving gene expression rather than the inability to attach or penetrate (Roizman et al., 1993).

Once initiated, CMV gene expression is regulated by a complex series of events at both the transcriptional and post-transcriptional levels. (Roizman et al., 1993). Protein synthesis in CMV is believed to follow a system of induction and repression that occurs in three phases: α , β and γ (Dimmock and Primrose, 1994). The designation of CMV genes as α, β and γ is based on sensitivity of gene expression at each phase, and the inhibition of DNA replication by inhibitors specific for viral DNA polymerase (Roizman et al., 1993).

Unlike β and γ transcripts that appear to accumulate slowly in the course of infection, α transcripts are observed immediately after the initial cellular infection (Roizman et al., 1993). The latter carry out the key regulatory functions including the control of viral replication (Dimmock and Primrose, 1994). Alpha (α) gene expression is under the control of a very strong transcriptional enhancer which responds to viral transactivators and cellular stimulation signals (Boshart et al., 1985). Because of the enhancer's ability to respond to the two stimuli, some researchers have suggested that α gene products play significant regulatory roles in persistence and latency of CMV as well as in viral reactivation (Roizman et al., 1993).

Beta (β) transcripts are expressed later in infection (Roizman et al., 1993). The first β products, are reportedly observed within 4 - 8 hours post infection and may persist up to 24 hours post infection (Roizman et al., 1993). The β genes are sensitive to DNA replication inhibitors indicating their dependence on viral replication (Dimmock and Primrose, 1994). The gamma (γ) genes encode structural proteins (Dimmock & Primrose, 1994). The γ gene products can be detected between 12 and 36 hours post-infection and may persist up to 24 - 48 hours post-infection (Roizman et al., 1993). γ classes are not sensitive to viral replication inhibitors indicating their independence on viral DNA replication (Dimmock and Primrose, 1994).

Genetic analysis findings have indicated that α products induce transcription of β genes; β gene products inhibit the expression of α genes and induce γ gene transcription through negative feedback mechanisms. Gamma (γ) gene products then inhibit β products (Roizman et al., 1993). Control mechanisms occur at both transcriptional and translational levels, however transcription of some of α and β genes takes place throughout the course of infection (Dimmock and Primrose, 1994). DNA replication is strictly

dependent on β gene products, including DNA polymerase and takes place by a rolling circle mechanism. (Dimmock and Primrose, 1994).

I.2.3 Establishment of Latency

Like other members of the family *Herpesviridae,* CMV has the ability to establish latency in the host (Roizman et al., 1993). The exact mechanism of establishment and maintenance of latency is not well understood. Clinically, CMV latency has been demonstrated by the appearance of CMV from endogenous sources and the transfer of infection through various organ transplants from asymptomatic seropositive to seronegative individuals (Chou S., 1986 and 1987). Activation of a latent CMV infection following therapeutic immunosupression as in cancer chemotherapy or transplantation is another reported common clinical observation of CMV latency (Roizman et al., 1993).

In vitro studies of the CMV latency have been hindered by the lack of an animal model susceptible to human CMV (Roizman et al., 1993). Genetic analysis studies by various investigators have indicated that transcription of the viral genome during latency is restricted to α and β genes (Dimmock and Primrose, 1994; Stevens J.G., 1989). Like HSV, latent CMV DNA is believed to exist as an episome (Dimmock and Primrose, 1994). As hypothesized by Dimmock and Primrose, latency seems to be maintained by a balance between the virus and the host's immune mechanism. The hypothesis is based on the observation in AIDS patients who in the presence of greatly decreased immune function are at high risk of developing life-threatening CMV illness. The exact mechanism of reactivation of a latent CMV infection in this group of patients is not well defined, however a number of theories are being considered. Robert Schooley (1990) suggests that the immunologic control of

CMV latent infection is mediated primarily by cellular mechanisms. Any defects in this arm of the immune system can lead to a reactivation of a latent CMV infection. Reported observations (Quinnan et al., 1985; Rook et al., 1985 and Blumberg at al., 1987) have shown defects in natural killer cell activity, in CMV-specific cytotoxic T lymphocyte effector function and in CMV-induced lymphocyte proliferation and interferon secretion are not uncommon in HIV seropositive individuals with advanced immune disease. Many such patients have evidence of a clinical CMV disease, consistent with reactivation. The loss of CMV-specific effector functions seem to occur in parallel with the decreases in other aspects of the cell mediated immune response as measured by the total number of circulating cells of the $CD4^+$ surface phenotype (Blumberg et al., 1987). *In vitro* studies using interleukin-2 as an immuno-modulating agent to restore CMV-specific immune defense mechanisms and prevent reactivation have been reported (Rook et al., 1983), however the relevance of these *in vivo* findings have not yet been addressed.

Peripheral blood mononuclear cells (PBMCs) have been documented as an important *in vivo* site for CMV latency (Schrier et al., 1985). In this study, nucleic acid hybridization techniques were used to locate RNA from the immediate early region of CMV DNA in circulating PBMCs of CMV seropositive (but asymptomatic) individuals. Because the individuals in the study had no evidence of clinical CMV disease, it was concluded that PBMCs with positive hybridization signals were in fact, harboring latent CMV genomes. In another study by the same group (Rice et al., 1984), monoclonal antibody (MAb) and immunofluorescence technology were used to demonstrate the presence of CMV antigens in PBMCs. Virus expression was again reportedly limited to immediate early genes. The PBMCs did not produce infectious viruses, nor were mature virions visualized by electron

microscopy. This observed restriction of gene expression in human CMV has also been described for mouse (La Femina and Hayward, 1986) and rabbit (DeMarchi, J.M., 1983) cell lines using the relevant animal viruses. In both cases there was no evidence of virus replication consistent with latent CMV infection. Similar observations have been reported by Braun et al. (1986), Einharn and Ost (1984), Nelson et al. (1990). These data all support the statement that CMV establishes a latent infection in the long-lived PBMC population of circulating leukocytes.

I.2.4 Reactivation

As suggested by a number of investigators (Dimmock and Primrose, 1994; Ho M., 1991; Roizman at al., 1993 and Schooley R.T., 1990) and as discussed above, a breakdown of the host's defense mechanisms leads to a reactivation of a latent infection to a symptomatic infection. As for latency, the exact mechanism of reactivation is yet to be determined. Reactivation is particularly common in AIDS patients and in most cases can lead to life and/or sight threatening complications (Schooley R.T., 1990).

Isolation of infectious CMV from peripheral blood cells is known to be associated with clinical CMV disease. Rinaldo et al. (1977 a and b) reported successful recovery of infectious CMV from both PBMCs and PMNLs from patients suffering from CMV mononucleosis. In another study (Gerna et al., 1992), infectious virus was detected in 45% of PMNL preparations of peripheral blood obtained from a cohort of solid organ transplant recipients. Their findings showed that PMNL and PBMC fractions from virtually all immunocompromised viremic patients were positive for both viral DNA and viral antigens, therefore suggesting that both leukocyte populations are consistently involved in viral infection, supporting observations made by

other investigators (Dankner et al., 1990). Further analysis indicated the occurence of some early events of the replication cycle in PMNLs. Using RNA PCR, they demonstrated the presence of an immediate early gene product (p72), in dextran - enriched PMNL fractions. Whether viral material detected in PMNL is entirely acquired by phagocytosis of infected cell debris in immunocompromised patients with a systemic CMV infection or represents acute infection of these cells is still controversial (Turtinin et al., 1987).

A previous report by the same group (Revello et al., 1992) demonstrated the presence of *de novo* synthesis of viral gene products in the PMNL fraction. Inoculation on human fibroblast cell cultures with preparations of infectious virus and dense bodies was followed 30 - 60 minutes later by accumulation of CMV lower matrix phosphoprotein (pp65) in nuclei of all infected cells, pp65 being the major structural component of dense bodies (Roizman et al., 1993). As a result of these findings, a hypothesis was put forward that the same phenomenon could occur *in vivo* after phagocytosis of infected cell debris by PMNLs and accumulation of pp65 from phagocytozed dense bodies into PMNL nuclei. The presence of viral components and infectious virus particles in PMNLs could well be due to two events: phagocytosis and *de novo* synthesis.

To date, there has not been a reported association of latency and PMNLs, the infectious virus in PMNLs is not uncommon in symptomatic clinical CMV disease (Roizman et al., 1993). Additional findings of infectious virus particles in PMNLs have been demonstrated by virus isolation from sonicated infected PMNL preparations (Wunderli et al., 1989) as well as by detection of a similar number of infected cell foci in cell cultures inoculated with infected PMNLs in the presence of absence of viral DNA inhibitors

(Revello et al., 1992). Electron microscopy studies have also shown the presence of complete virus particles in PMNLs (Martin et al., 1984).

I.3 Interactions Between HIV and CMV- a special case of reactivation

It was noted quite early in the epidemic of HIV infection that some of the immune abnormalities in AIDS patients had a superficial resemblance to abnormalities observed in patients with primary CMV infection (Schooley R.T., 1990). Such abnormalities included the observed changes in the relative distribution of lymphocyte surface phenotypes in the peripheral blood and a decrease in specific responses to mitogens and antigens (Rinaldo et al, 1980, Carney et al, 1981 and Reinherz et al, 1980). These superficial similarities and the fact that the vast majority of gay and bisexual men are seropositive for CMV led to early speculation that it may in fact may have been the etiologic agent of AIDS. The proposed theories of CMV and HIV interaction at different levels are summarized below.

1.3.1 Immune mediated mechanisms

A clear understanding of the effects of viruses on host immune function and mechanisms of immune containment of viral infection in general are important in describing the potential immunologic bi-directional interactions of CMV and HIV. Both humoral and cell mediated immunity (CMI) are elicited following primary CMV infection (Rook A.H., 1988). The importance of humoral immunity is likely to be very small since even in fatal CMV infection, antibody responses have been shown to remain intact (Simmons et al., 1977).

CMV has general immunosuppressive effects on the host (Griffiths and Grundy, 1987). The exact mechanism of immunosuppression is not completely understood. It has been noted that acute CMV infection is associated with added susceptibility to bacterial and fungal infections (Rand et al., 1978 and Rubin et al., 1977). In CMV mononucleosis, mononuclear cells show a decreased blastogenic responsiveness to a variety of mitogens (Rinaldo et al., 1980). In addition, the ability to generate new cytotoxic T-cells is impaired during this illness (Carney et al., 1983).

The effects of HIV on the host's immune system have been the focus of intense research in the last decade and are reviewed in great detail by Levy (1994). The reported abnormalities involve every arm of the immune system and include decreased blastogenic response to mitogens and antigens (Ciobanu et al., 1983), decreased cytotoxic lymphocyte responses (Rook et al., 1983) and defective natural killer cell numbers and cellular function (Ljuggren et al., 1989) as well as impaired Antibody Dependent Cellular Cytotoxicity (ADCC) (Tyler et al., 1990).

In AIDS, it is clear that HIV-induced decrease in CMI is central to the development of CMV disease (Schooley R.T., 1990). In a concomitant active infection with CMV and HIV, additive immunosuppression is a likely major contributing factor to the severity of the immune disease (Roizman et al., 1993). Dysfunctional CMI induced by both viruses could possibly lead to a worsening immune deficit allowing enhanced viral replication of both HIV and CMV and a worsening of clinical disease induced by one or the other.

I.3.2 Phenotypic Mixing

CMV and HIV, both enveloped viruses, can infect the same cell type *in vivo* (Zavada J., 1982). This observation raises the possibility of phenotypic

or genotypic mixing resulting in the formation of pseudotype or recombinant viruses. As stated previously, a large percentage (60 - 100%) of AIDS patients have evidence of CMV infection (Carney and Hirsch, 1981). Although both viruses infect monocytes and lymphocytes, CMV generally mounts a latent infection in these cells with gene expression limited to immediate early (IE) antigens without the production of infectious virus (Dimmock and Primrose, 1994). Immunocytochemical studies have demonstrated the expression of HIV antigens on endothelial cells, despite the fact that these cells are generally non-permissive for HIV infection *in vitro* . As CMV may infect such cells, we may be observing phenotypic mixing between CMV and HIV to explain the presence of HIV antigens in these cell types (Pomerantz et al., 1987).

I.3.3 Intracellular mechanisms

CMV viral gene products can act directly on the HIV LTR region or indirectly via an interaction with cellular products. (Mosca et al., 1987). *In vitro* studies utilizing an HIV LTR linked to a reporter gene have demonstrated transcriptional activation of the HIV promoter by CMV proteins acting in *trans* (Koval et al., 1991 and Biegalke et al., 1991). Further mutational analysis studies suggest the TATA box of the HIV LTR is the target region for CMV transactivation (Koval et al., 1991).

Under usual circumstances, HIV transcription is induced by cellular factors. One of the cellular factors is nuclear factor - kappa B (NF-kB) (Lenardo and Baltimore, 1989). In the majority of human cells, NF-kB is found in the cytoplasm bound to an inhibitor - IkB. Upon activation of cytoplasmic NF-kB, the IkB complex is phosphorylated by protein kinase C, leading to a dissociation of IkB and NF-kB (Baeurle and Baltimore, 1988). NF-kB then translocates to the nucleus where it binds to U3 of the HIV LTR

region, generally referred to as the NF-kB binding region. CMV virion-associated kinases could potentially trans-activate HIV LTR through IkB phosphorylation (Stanat et al., 1991).

HIV has also been reported to up-regulate CMV. *In vitro* studies have demonstrated the ability of HIV *tat* to transactivate the CMV immediate early gene (Kim and Risser, 1993), raising a possibility that a bi-directional positive feedback mechanism exists between HIV and CMV. The *in vivo* significance of these observations is unknown. An overall model for the interaction of HIV and CMV is shown in figure 1.

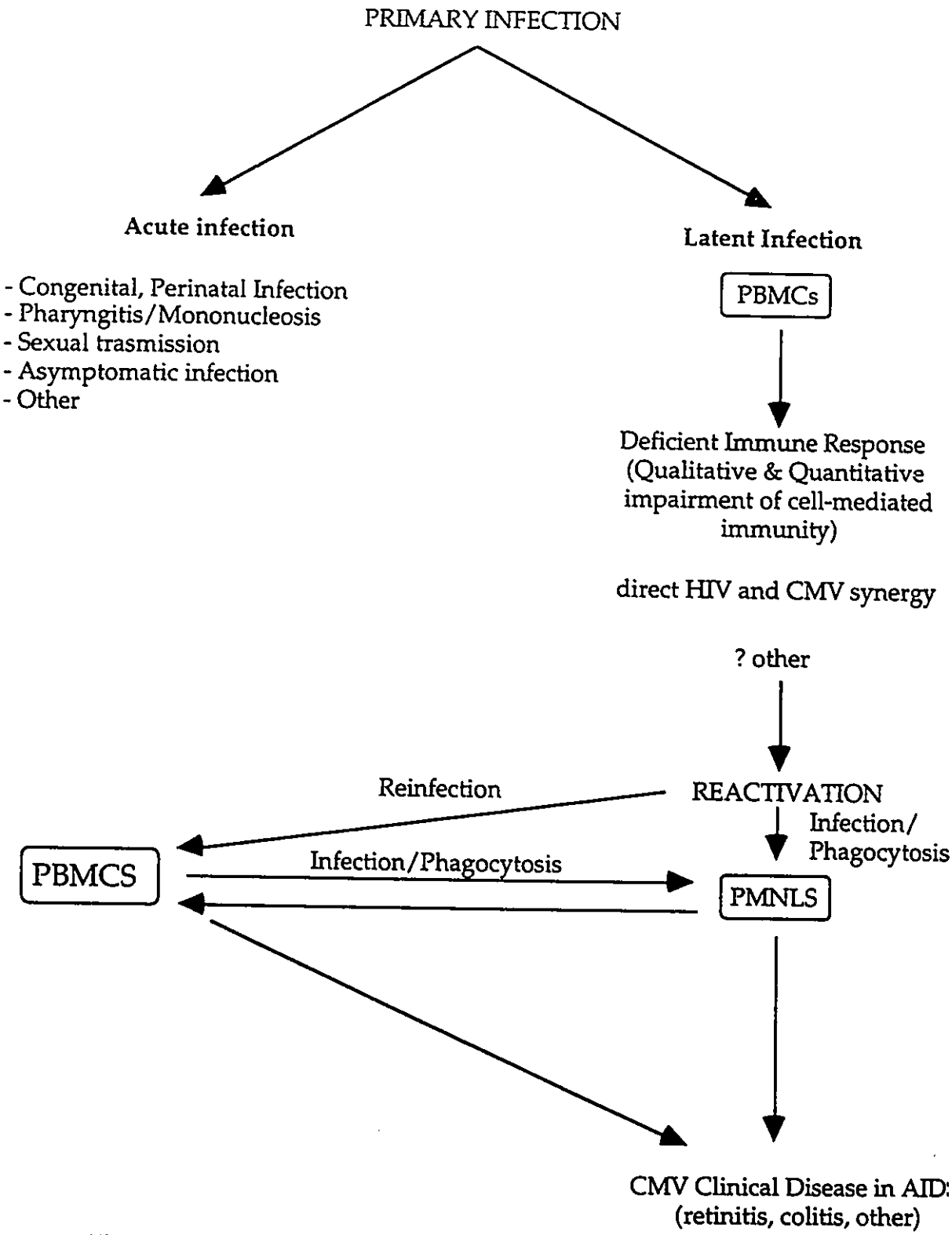


Fig 1. Diagrammatic representation of CMV latency and reactivation using HIV infection as a specific example.

I.4 Pathogenesis of CMV infection

I.4.1 Acute infection

Epithelial cells are most commonly involved in the pathogenesis of acute CMV infection (Roizman et al., 1993). The two major tissues involved are the kidneys (from which a prolonged shedding of the virus into the urine is very common) and the salivary glands (Stevens J.G., 1989). The latter observation makes saliva one of the principal means of CMV transmission in the population (Roizman et al., 1993). In acute infection, a mononucleosis - like syndrome is common with symptoms such as fever, sore throat and abnormal liver function due to hepatic involvement (Hoeprich et al., 1994). The immune status of the host in acute infection determines the extent of organ involvement (Roizman et al., 1993).

I.4.2 Latency and reactivation

The lack of an animal model for CMV latent infection has limited the extent of our knowledge in this field. It is evident that the virus establishes latency in most individuals after initial infection (Roizman et al., 1993). Most of the general population that harbors latent CMV, have no reported clinical symptoms of the infection as long as their cellular immune system is intact. Thus, symptoms often protean in nature, will only occur in immunocompromised individuals.

I.4.4 CMV infection in AIDS patients

Thus, patients with advanced AIDS are at significant risk of developing CMV disease. Clinical manifestations in these patients are quite variable and have been reported to include interstitial pneumonitis, hepatitis, esophagitis, colitis, pancreatitis and chorieretinitis (Jordan et al., 1994; Schooley R.T., 1990).

Fever and respiratory problems (especially pneumonia) are common (Roizman et al., 1993). CMV retinitis is the another most common form of clinical CMV disease in AIDS patients. The disease presents itself as a painless progressive loss of vision. One eye initially may be involved but if it remains untreated, disease usually progresses to both eyes leads to irreversible blindness (Schooley R.T., 1990). In general CMV disease is only seen in most advanced AIDS, when the CD4 cell count is below 100/μl (Roizman et al., 1993).

I.5 Therapy for CMV infection in AIDS patients

The efficacy of therapy for CMV infection is reduced by the presence of severe immune dysfunction in AIDS patients Schooley R.T., 1990). The currently approved treatment for CMV disease are ganciclovir and foscarnet (Jordan et al., 1994). In most cases, ganciclovir is the initial drug of choice for treatment (Jordan et al., 1994).

I.5.1 Ganciclovir

Ganciclovir is an acyclic analogue of guanine, acting by inhibiting viral DNA synthesis (Spector S.A., 1991). In CMV infected cells, the drug is phosphorylated to its monophosphate and then to an active triphosphate derivative possibly by a virally-coded deoxyguanosine kinase (Stanat et al., 1991). Inhibition of viral replication is competitive, with the triphosphorylated form of ganciclovir competing with dGTP for DNA polymerase. Successful inhibition leads to inhibition of DNA synthesis and hence termination of the DNA elongation process (Jordan et al., 1994).

Ganciclovir is administered intravenously at a dose of 5mg/kg every 12 hours (Jordan et al., 1994). In 80-100% of AIDS patients, intravenous

administration at this dose leads to stabilization or resolution of retinitis especially if the drug is administered early in the course of infection (Jordan et al., 1994). Long term administration of ganciclovir in AIDS patients is usually required as relapses are universal once therapy is stopped. Early administration of drug is therefore important for a successful outcome. Recently, very early use of ganciclovir was reported to prevent CMV disease after bone marrow transplantation in certain patients at high risk of CMV disease due to prolonged immunosuppression (Pomeroy and Jordan, 1994). Ganciclovir was administered to patients when cultures of their bronchoalveolar lavage specimens or blood yielded CMV shortly after transplantation. The result was a decreased incidence of serious CMV disease and significantly improved survival rates. These findings of therapeutic efficacy when ganciclovir was administered early underscores the need for a specific, sensitive and rapid diagnostic technique that can distinguish between latent and active infections such that patients who could benefit from early intervention can be identified (Schooley, R.T., 1990). This could be particularly beneficial in AIDS patients.

I.5.2 Foscarnet

Foscarnet is a pyrophosphate analogue that acts by inhibiting viral DNA polymerase (Jordan M.C., 1994). It is one of the drugs licensed for the treatment of CMV retinitis in AIDS patients. The major side effects of foscarnet include nephrotoxicity and hypercalcemia (Jordan M.C., 1994).

I.6 Diagnosis of CMV Infections in AIDS patients

Although much progress has been made in identifying the importance of CMV as an opportunistic pathogen in AIDS patients, CMV diagnosis is still

hindered by the lack of sensitivity of most of the currently available techniques. CMV diagnosis is further complicated by the presence of latent CMV infection in the general population, with up to 80% of adults having been exposed to CMV during their lifetime (Roizman et al., 1993). In broad terms, currently availabie techniques can be grouped into six major categories: a) serology b) cell culture c) antigen detection techniques d) nucleic acid hybridization and e) nucleic acid amplification. Viral isolation in tissue culture remains the gold standard in establishing the diagnosis of CMV infection (Baskin and Jordan, 1994). Advantages and limitations of viral isolation and other diagnostic techniques commonly used in CMV diagnosis will be discussed in detail.

I.6.1 Serology

CMV infection can be diagnosed serologically either by a demonstration of seroconversion or by detection of IgM antibodies (Baskin and Jordan, 1994). This approach has a very limited utility in AIDS patients given the dyseregulation of B cell function resulting in polyclonal expansion of immunoglobulin production in late HIV-related immunodeficiency (Baskin and Jordan, 1994). In addition, IgM antibodies can be produced for extended period of times after primary CMV infection and heterotypic IgM responses have also been reported (Baskin and Jordan, 1994). Serologic tests are still useful in studies of the epidemiology of CMV infection in the community and in the evaluation of vaccines in the future.

I.6.2 Cell Culture

Isolation of viruses in cell culture (CC) is based on the concept that each virus has an affinity for growth *in vitro* in a specific cell type. Generally,

viruses differ with respect to types of cell cultures in which recovery and identification may be successfully achieved. CMV can be recovered from body fluids - blood, urine, saliva, tears, milk, semen, vaginal or cervical secretions. However, urine and blood are the samples most commonly processed in diagnostic laboratories (Paar and Strauss, 1992). *In vitro* , CMV can be grown in diploid cell cultures. Commercially-available human fibroblasts are the most commonly used. Briefly, the fibroblasts are inoculated with a patient's sample and incubated for up to six weeks. Microscopic observation for the characteristic CMV cytopathic effect is usually performed twice weekly during the incubation period, with a positive result taken as evidence of active CMV replication.

The main advantage of cell culture as a diagnostic tool is its ability to identify infectious virus. The technique has many limitations. The time required for growth and identification is too long to be of optimal use in patient management (Yolken R.H., 1993). Depending on the amount of CMV present in the specimen, recovery of the virus can take up to 6 weeks. The technique requires fresh specimens as freezing and thawing have adverse effects on viral yield (Chou S., 1990). The maintenance of tissue culture is costly and technically demanding. Moreover, the isolation of virus can be prevented by overgrowth of other rapid growing microorganisms such as herpes simplex virus and fungi which can destroy cell monolayers (Chou S., 1990).

The Shell Vial Assay (SVA) represents a method of improving the diagnostic yield of cell culture (Yolken R.H.., 1993). In SVA, centrifugation is used to enhance viral infection on monolayers prior to incubation. At appropriate time points, the cell preparations are stained with fluorescent antibodies to virally-coded proteins (Gleaves et al., 1984). The antigen is

usually detected prior to the development of the characteristic cytopathic effect of CMV in cell culture. Like cell culture, SVA has some limitations. The sensitivity of the assay depends on the specimen type, the number of vials inoculated, the type of antibodies used, the incubation time prior to staining, the type and age of cells used, the concentration of virus in the specimen and the length of time cultures are maintained (Miller et al., 1994).

I.6.3 Antigen Detection Techniques

The availability of a wide range of antigen-specific monoclonal antibodies (MAb) has generated a great interest in the use of antigen (Ag) detection techniques in monitoring CMV disease (van der Bij et al., 1990). The most commonly used technique is the enzyme linked immunoassay (ELISA) based on the direct detection of CMV antigen in the specimen. In this assay, the antibody is adsorbed onto polystyrene microtiter wells and the immobilized antibody is used to capture the antigen in the specimen (Baskin and Jordan, 1994). If the target antigen is present, it will bind to the immobilized antibody. A second antibody coupled to an enzyme, will then be added followed by a calorimetric enzyme substrate. The technique is rapid, quantitative and can be performed on stored samples. The interpretation of the assay results can sometimes be complex, especially if the signal is weak. In particular, false negatives can be reported if the MAbs generated to specific strains of CMV fail to recognize the corresponding Ag in a given clinical specimen (Chou S., 1990). In a study of 21 distinct CMV strains, two strains were reportedly not recognized by the immediate early MAb that was used (Chou and Scott, 1988). In addition, it is not clear if this assay can reliably distinguish between active and latent infection (Chou and Scott, 1988).

I.6.4 Nucleic Acid Hybridization

The increase in availability of cloned CMV sequences has allowed us to consider the use of nucleic acid hybridization in the CMV diagnosis in AIDS patients (Chou S., 1990). Although the technique is rapid and the results can be obtained within 24 hours and is commercially available, it is still technically demanding. In addition, hybridization procedures can detect nucleic acids that are not being transcribed into proteins and thus can not differentiate latent and active infections (Yolken R.H., 1993). The lack of signal or target amplification also limit their sensitivity when low viral loads are present in the specimen. Sensitivity as low as 29% has been reported in some early work (Chou, S., 1983).

I.6.5 Nucleic acid amplification

The description of DNA amplification using the polymerase chain reaction (PCR) by Saiki et al. (1988) has had vast implications for the diagnosis of viral infection. During the cyclic, primer-driven process of target amplification, 10^6 - 10^7 new copies of a given nucleic acid sequence are generated making PCR the most sensitive assay yet designed. The target nucleic acid which determines the sequences of oligonucleotides complementary to their annealing sites provides specificity of PCR reactions. Despite its requirement for specialized equipment and the required attention to detail to avoid contamination, the high sensitivity and specificity of PCR makes it potentially useful for the detection of DNA present in very low copy numbers (Persing et al., 1993). Depending on the product detection assay utilized, PCR results can be available in 6 hours. Unlike cell culture, the procedure is less technically demanding and can be performed on stored samples. Although PCR can not differentiate live from dead or latent from

actively replicating viruses, its high sensitivity is potentially advantageous. Specific applications may also be designed to overcome its limitations.

I.6.6 Comparative studies

A number of investigators have reported comparative studies on different available techniques for the diagnosis of CMV infection in immunocompromised individuals.

In a study by Samra et al. (1994), SVA and CC were compared to the IgM antibody assay. Fourteen percent of the patients tested were reported to be positive for CMV by SVA compared to only 3% by CC. The discrepancies in positive specimens by the two techniques was interpreted to relate to the increased sensitivity of SVA. IgM was only detected in 56% of culture-positive specimens indicating the higher sensitivity of the latter.

Miller et al. (1989) compared the sensitivity and specificity of CC, SVA with that of the polymerase chain reaction (PCR). The sensitivity of CC and SVA compared to PCR was 67% and 79% respectively. The observed differences among PCR, CC and SVA results were likely due to the lack of sensitivity of SVA and CC compared to PCR. These findings are in agreement with other more recently published reports comparing PCR with CC, SVA or antigenemia (Einsele et al., 1991; Gerna et al., 1991 and Jiwa et al., 1989). PCR was positive earlier and remained positive longer than other CMV detection methods. In addition, PCR did predict a risk of relapse - CMV DNA was detected by PCR and successfully correlated with viremia and clinical disease in patients with primary CMV infection as well as reactivated infections (Brytting et al., 1992).

In another study by Sandin et al. (1991), sequential dilutions of infected cell lines was utilized to study the sensitivity of CC, SVA and PCR. A serial dilutions of crude cultures harvests from 2 CMV strains, Towne and a clinical

urine isolate were made up to 1: 10^6. Ten microlitre aliquots of the original sample and each dilution were tested by CC, SVA and PCR. On average PCR was 100X more sensitive than evaluation of the characteristic CMV cytopathic effect (CPE) in CC and the predictive value of negative SVA result was lower than that of PCR.

In the past 8 years, a number of studies have reported the usefulness of PCR in the diagnosis of CMV infection with emphasis on the sensitivity and specificity it offers (Cassol et al., 1989; Demmler et al., 1988; Hsia et al., 1989; Shibata et al., 1988; Van den Berg et al.,1989). A study by Hsia et al (1989) compared the sensitivity of PCR and CC in detecting CMV clinical isolates in peripheral blood cells and urine samples of immunocompromised patients. Subgenomic CMV could be selectively amplified between 10^5 - 10^6 fold after 25 cycles of PCR with Taq polymerase. In 37 urine samples analyzed simultaneously by CC and PCR, identical results were obtained in 35 samples, while 2 tested positive only by PCR.

The significance of PCR for the early diagnosis of CMV infection has also been documented (Cassol et al., 1989). A sensitive PCR protocol was utilized to establish a diagnostic assay for the identification of CMV immediate early sequences in clinical specimens. PCR was found to be highly CMV - specific, recognizing both wild-type and laboratory strains of CMV. There was no reported cross-reactivity with human DNA or DNA from other herpes viruses. Using cloned CMV AD 169 *Eco* RI fragment -J as a template, the sensitivity of the assay was established to be 1 viral genome per 40 000 cells.

In a study by Shibata et al (1988), two separate CMV genes were amplified to examine 28 tissue culture isolates. The IE oligomers used were complementary to the gene coding for the predominant protein found early

in infection whereas the LA oligomers were complementary to the gene coding for a structural glycoprotein present in large amounts in infection (Roizman et al., 1993). All of the 28 isolates were found to contain CMV, although 2 of the isolates were positive for only one of the two genes. CMV PCR was more sensitive than the standard culture assay. Viral genomes from as few as 1 CMV plaque - forming unit (PFU) could be detected by PCR.

The clinical significance of PCR for the detection of CMV DNA in immunocompromised patients has been documented. The presence of CMV DNA in blood specimens of immunocompromised patients and its clinical significance was investigated by PCR (Zipeto et al., 1992). A total of 293 blood samples from 86 immunocompromised patients were subjected to PCR and assays for viremia and antigenemia. Of the 86 patients, 23 underwent clinical and virological follow-up for CMV infection. Results were concordant between PCR and assays for viremia and antigenemia in 124 positive and 110 negative samples, while 59 samples were positive by PCR alone. Most of the 59 samples were reportedly from patients with diagnosed CMV infection. Indeed, 54 (91.5%) samples were from patients with virologic or clinical signs of CMV infection preceding or following blood collection. PCR in this case was more sensitive and specific than the other two assays with about 30% of positive specimens detected as positive for CMV by PCR alone.

Long-term clinical and virologic follow-up was initiated in one patient. Sixteen days after kidney transplantation with repeatedly positive PCR results, the patient presented a first peak of viremia and antigenemia as well as clinical symptoms of CMV. Following treatment with ganciclovir, viremia and antigenemia became negative at day 61 while PCR remained positive. A second peak of viremia and antigenemia associated with CMV disease appeared at day 82 when a second course of ganciclovir treatment was

initiated. It is interesting to note that in contrast with viremia and antigenemia assays, PCR remained positive throughout and may have allowed us to predict the observed early relapse.

In another recent study the presence of CMV DNA in serum was shown to precede the development of CMV clinical disease (Hansen et al., 1994). Five consecutive serum samples were examined from 52 HIV-seropositive patients, 19 of whom had a clinical diagnosis of CMV retinitis. The findings showed that 11 patients who developed CMV retinitis had positive CMV DNA results 3 months before the diagnosis of clinical disease. Another 3 retinitis patients who initially tested negative for CMV DNA by PCR, became positive with the onset of clinical retinitis.

I.7 Hypothesis

There is strong evidence (Rice et al., 1984; Schrier et al., 1985) that supports the fact that CMV establishes a latent infection in the long lived mononuclear cell sub-population of circulating leukocytes – PBMCs. The presence of infectious CMV in PMNLs may reflect either phagocytosis by or infection of this sub-population by replicating virus spilling from reactivated sites of previously latent infection. If so, one can postulate that a highly sensitive DNA detection technique such as PCR performed on the appropriate circulating leukocyte sub-population may indicate latent infection should the PBMCs be positive and PMNLs negative whereas a positive signal in PMNLs indicates active replication and may correlate with or predict impending clinical disease.

I.8 Objectives

The overall objective of this work was to develop a sensitive and specific PCR protocol to monitor CMV disease status in HIV-seropositive patients. Emphasis was placed on the application of PCR for the detection of CMV genomic DNA in PBMCs and PMNLs and correlation of the results with CMV disease status in the study population.

In light of the advantages and limitations of the different techniques for the detection of CMV in clinical samples, PCR is an ideal choice for viral detection regardless of strain or copy number, as long as primers are selected to amplify a highly conserved region of the CMV genome. Our approach was (i) to utilize a highly sensitive and specific PCR protocol for the detection of CMV genomic DNA (ii) to use the designed protocol for amplification of CMV DNA in circulating leukocyte sub population, i.e. PBMCs and PMNLs to ensure differentiation of latent and active infection, and (iii) to prospectively study the role of the optimized assay in the monitoring of CMV disease status in HIV-seropositive individuals, selected as those at highest risk of clinical CMV disease.

II. MATERIALS AND METHODS

II.1 Patient Population and Clinical Specimens

Anticoagulated heparinized blood samples were collected from 25 different HIV seropositive individuals seen as ordered by clinicians at the Ottawa General Hospital Immunodeficiency Clinic. The samples were subjected to conventional culture as well as PCR for the detection of CMV, as detailed below. Heparinized blood specimens from five HIV-seronegative, healthy volunteers were also obtained and only subjected to PCR.

II.1.1 Leukocytes Separation

Blood leukocytes were isolated from heparinized whole blood by dextran sedimentation by a procedure modified from that of van der Bij et al (1988). Briefly, a maximum of 8 ml of blood was spun down at 400 x g (using an IEC Centra-8R containment centrifuge (International Equipment Company, Massachusetts, USA) for 15 minutes. An aliquot of 1.5 ml of plasma was saved and stored at -70°C. The remaining blood was diluted to 8 ml with 1 x PBS and layered carefully on 5 ml of a sodium metrozoate, Dextran 500 solution (Polymorphprep™, Nycomed Pharma AS, Norway) in a 15 ml centrifuge tube. Following centrifugation (without the brake) at 450g for 35 minutes at 22°C, two leukocyte bands, clearly visible, were isolated. The top band (consisting of PBMCs) and the lower band (consisting of PMNLs) were transferred to separate fresh labeled tubes. The PMNLs were diluted by addition of one volume of 0.45% sodium chloride solution in order to restore normal osmolarity. Both cell pellets were then washed once in 1 x PBS (made up to 10 ml) and centrifuged at 400g (22°C) for 15 minutes. The cells were pelleted and the remaining erythrocytes were lysed by addition of 0.8%

ammonium chloride (BDH Chemicals, Mississauga, Canada), followed by two washes with 2 ml 1 x PBS (400g, 10 minutes, 22°C). Both leukocyte fractions were counted , with live cells enumerated by Trypan blue dye exclusion. 50 µl of the sample was mixed with 50 µl of Trypan blue in a 0.5 µl eppendorf tube. Approximately 50 µl of the mixture was pipetted onto the hemocytometer , enough to cover the counting squares. The cells were counted under a light microscope, followed by a 10 minutes spin at 4000 rpm. After the spin, the supernatant was discarded and the cells resuspended in 1 ml 1XPBS and spun down (4000 rpm) for 5 minutes. The cell pellets were stored at -70^0 C after discarding the supernatant after the spin.

II.1.2 Cell Culture of Clinical Specimens

MRC-5 fibroblasts were inoculated with at least 1 x 10^6 PMNLs and grown in with Eagles minimal essential medium (MEM) with 10% fetal calf serum (FCS), adjusted to the appropriate volume. The number of PMNLs inoculated was dependent on the quantity recovered from the specimen. A maximum of 2 x 10^6 PMNLs was inoculated into a single 15 ml polystyrene flask (Costar, Massachussets, USA) to avoid the toxicity to the cell monolayer occasionally seen when that number is exceeded (Buller et al, 1992). After centrifugation at 700g for 45 minutes, 2 ml of viral culture medium was added to the flask, which was then incubated at 37°C and observed by light microscopy for typical virus-induced CPE twice weekly for up to six weeks.

II.1.3 DNA Extraction

DNA was extracted from PBMCs and PMNLs using phenol and guanidine thiocyanate monophase solution (TRI REAGENT™ TR-118, Molecular Research Centre, Inc., Cincinnati, Ohio). The isolation was accomplished by the single step liquid-phase separation and performed

according to manufacturer's recommendations. The cells were homogenized in 0.8 ml of TRI REAGENT™ supplemented with 1% Microcarrier™-Gel-TR (to facilitate RNA isolation), then incubated at room temperature for five minutes. Following the addition of 0.2 ml of chloroform (vortexed and stored at room temperature for 15 minutes), the homogenate was centrifuged at 12 000g for 15 minutes at 4°C and separated into three phases: aqueous, interphase and organic. DNA remained exclusively in the interphase, RNA in the aqueous phase and proteins remained in the organic phase. DNA was precipitated from the interphase with 0.3 ml of 100% ethanol, incubated at room temperature for three minutes and sedimented by centrifugation at 2000g for five minutes at 4°C. Following three washes of the DNA pellet in 0.1M sodium citrate in 10% ethanol at room temperature for 30 minutes (with periodic mixing), the suspension was centrifuged at 2000g for five minutes at 4°C and the DNA pellet suspended in 75% ethanol stored at room temperature for 20 minutes, then centrifuged at 2000g for five minutes. After being dried briefly under vacuum, the DNA pellet was solubilized in 8 mM NaOH (0.5 ml/10^7 cells) and the DNA-containing solution was transferred to new tubes. The pH was adjusted to 8.4 using 0.1M HEPES (free acid) and the DNA sample was stored at -70°C until further use.

II.2 Primer Design

Early hybridization studies using different CMV probes showed broad cross reactivity between different CMV strains in both late and early segments of the CMV genome (Demmler et al, 1988 and Shibata et al, 1988). For initial primer design, the genome sequences of human CMV available in the literature up to 1988 were used. The sequences for IE and LA primers were as published by Shibata et al, 1988 and Demmler et al, 1988 respectively. The two

primers sets represent conserved sequences on CMV genome present during early and late infection.Using this published information, two sets of primers (IE and LA) and a complementary chemiluminescent LA probe were synthesized by a commercial manufacturer (National Biosciences, Plymouth, MN). Human β-actin gene primers (National Biosciences, Plymouth, MN) were also used in our work, to ensure the presence of amplifiable DNA in all samples (see below). The sequences of all primers and probes are shown in Table 1.

II.3 Polymerase Chain Reaction

Optimal conditions for the performance of each PCR protocol were established by initial standardization experiments. For this work, a range of primer and $MgCl_2$ concentrations were evaluated to determine under which conditions a standard positive control (CMV strain AD169) was best amplified. The optimized protocols were used in all subsequent experiments. Each PCR reaction tube contained the following reagents in a total volume of 50 µl: DNA extracted from 100,000 cells; deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP) at 200 mM per nucleotide (Perkin-Elmer Cetus, Norwalk, CT); Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) at 2U/reaction; IE primers at 0.4 µM or LA primers at 1.2 µM, Tris-HCl (pH 8.3) adjusted to 10 mM; KCl 50 mM; $MgCl_2$ 1.5 mM; H_2O to 50 µl. For amplification reactions utilizing IE primers, the samples were overlaid with 60 µl of mineral oil to prevent evaporation. This was not necessary for reactions utilizing LA primers, due to performance of the assay in a different model of thermal cycler (see below). As a control of false negative results (due to the absence of amplifiable DNA or the presence of PCR inhibitors in the sample), β-actin primers (BA-1 and BA-2) were added to all reactions at a final concentration of 0.5 µM to amplify the β-actin gene present in all human cells. To decrease the amplification of nonspecific products, components and reaction tubes were kept at 4°C during the preparation of the reaction mixture.

II.3.1 Positive and negative PCR controls

CMV strain AD169 (ATCC VR-538, Rockville, MD) was grown in MRC-5 (Whitaker, MN) tube cultures until a cytopathic effect (CPE) was observed in 50 to 75% of the infected monolayer (3+ CPE). Briefly, tubes containing MRC-5 cells were inoculated with 0.1 ml of AD169 stock (3×10^6 PFU/ml), incubated

at 37°C with 5% CO_2 and evaluated microscopically every other day for up to six weeks to determine development of CPE typical for human CMV (Apperly and Goldman, 1988). At 3+ CPE (75%), the tubes were scraped. The cell suspensions from the tubes were clarified by centrifugation. The supernatants were then pooled, aliquoted (0.1 ml/vial) and frozen at -70°C until use.

Uninfected MRC-5 cells, PBMCs, PMNLs as well as DNA from Herpes Simplex virus types 1 and 2, Epstein Barr Virus (EBV) infected Raji cells (courtesy of Dr. F. J. Diaz-Mitoma, Research Institute at Children's Hospital of Eastern Ontario), and human placenta (Perkin-Elmer Cetus, Norwalk, CT) were used as negative controls. Chromosomal DNA from a *Neisseria gonorrhoeae* isolate MSII-MS (courtesy of Dr. J. A. Dillon, Department of Microbiology and Immunology, University of Ottawa) was also used as negative controls.

II.3.2 PCR Cycle

Amplification reactions utilizing IE primers consisted of 39 cycles of denaturation (94°C x 2 minutes), annealing (55°C x 2.5 minutes) and extension (72°C x 1.5 minutes) performed in a DNA thermal cycler (Model 480, Perkin-Elmer, Norwalk, CT). To ensure all primers were extended, an

Table 1: Primers and Probes used in PCR protocols

Primer/Probe	Sequence (5′ to 3′)	Product Length (bp)[b]
Primers:		
IE-1[a]	CCACCCGTGGTGCCAGCTCC	160
IE-2[a]	CCCGCTCCTCCTGAGCACCC	
LA-1[a]	CACCTGTCACCGCTGCTATATTTGC	400
LA-6[a]	CACCACGCAGCGGCCCTTGATGTTT	
BA-1[a]	TGACGGGGTCACCCACACTGTGCCCATCTA	661
BA-2[a]	CTAGAAGCATTGCGGTGGACGATGGAGGG	
Probes:		
LA	GTCGCCTGCACTGCCAGGTGCTTCG	

a – Previously published (Demmler et al., 1988 and Shibata et al., 1988)

b – Length of the product obtained following amplification using the indicated primer pair

additional cycle was run with the same denaturation and annealing parameters, and a final seven minute extension step. Following PCR, the reaction mixture was separated from the oil layer and stored at -20°C until further processing.

For LA primers, amplification was performed in a more advanced DNA thermal cycler (Turbo 9600; Perkin-Elmer, Norwalk, CT) and consisted of 35 cycles of DNA denaturation (94°C x 40 seconds), annealing (65°C x 90 seconds) and extension (72°C x 120 seconds). After a final ten minute extension, amplified products were removed and stored at -20°C for later detection.

II.3.3 Prevention of contamination

A major drawback of PCR is the potential for contamination. In theory, because of its high sensitivity, the introduction of a single target in any given sample can yield up to 10^6 molecules after 30 cycles thus giving a

false positive result (Persing et al., 1993), the most common source of contamination being previously amplified DNA. Extreme precautions are necessary to prevent this problem from occurring. To avoid possible contamination of PCR mixtures, all reactions were performed under stringent conditions following the recommendations of Kwok and Higuchi (1989). These precautions included the use of sterile latex gloves, sterile pipette tips and tubes, aerosol barrier pipette tips, sterile reagents divided into aliquots and a separate set of pipettes in separate rooms for nucleic acid extraction, the pre-PCR handling of specimens and PCR product analysis steps. In addition, negative controls were run along with the test samples for all reactions. These included an aliquot of reaction mixture with and without addition of a DNA sample known not to contain CMV sequences.

II.4 Southern Blot Hybridization

II.4.1 Transfer

PCR product was detected by direct gel analysis, Southern transfer and hybridization. An aliquot of the amplified products (10 µl plus 3 µl of 1 x Bromophenol blue) was electrophoresed at a constant voltage of 100 V for 1.5 hours through a 2% agarose gel (Ultra PURE Agarose, Life Technologies, Inc., Maryland, USA) stained with ethidium bromide (1 mg/ml) in 1 x TBE buffer. The presence of bands of the appropriate size was determined by photography under UV illumination with Polaroid Type 57 High Speed Film (Polaroid Co., Cambridge, MA). The gels were incubated in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 seconds, then rinsed briefly in water. This was followed by a 30 minute incubation in neutralization solution containing 1 M Tris (pH 7.4), 1.5 M NaCl. The denatured DNA was then transferred by capillarity using 20 x SSC (0.3 M Na Citrate and 3 M NaCl, pH 7.0) onto a pre-

soaked BIOTRANS nylon membrane (ICN Biomedicals, Inc., Ohio, USA). After overnight transfer, the membranes were rinsed gently in 2 x SSC, air dried and the DNA cross-linked onto the membrane by exposure to UV irradiation on a transluminator for three minutes.

II.4.2 Hybridization Reaction

The membranes were incubated in hybridization mixture containing 5 x SSC, 0.3% blocking buffer™, (Blocking reagent™, Boehringer Mannheim, Montreal, Canada) 0.1% sarcosyl and 0.01% SDS at 42°C for two to three hours with 10-20 ml of probe, depending on the number and sizes of membranes. Following an overnight hybridization, the unbound probe was removed by the following washes:

- 2 x 5 minutes at room temperature with 2 x SSC and 0.1 % SDS
- 1 x 45 minutes at hybridization temperature (42°C) with 0.5 x SSC and 0.1% SDS
- 1 x 2 minutes at room temperature in 100 mM Tris (pH 7.4) and 150 mM NaCl
- 1 x 45 minutes at room temperature with 2% blocking buffer™ in 100 mM Tris (pH 7.4) and 150 mM NaCl
- 2 x 5 minutes at room temperature in 100 mM Tris (pH 7.4) and 150 mM NaCl
- 1 x 2 minutes at room temperature with 100 mM Tris (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$

The hybridization membranes were then blot dried with Whatman 3MM Filter paper and sprayed with Lumi-phos 530 substrate in aqueous buffer (Cambridge Research Biochemicals, USA). To prevent drying, the membranes were wrapped in plastic, and were then exposed to Kodak X-ray

film at 37°C for 1.5 hours. Quantitation of bands was done using an Enhanced Laser Densitometer (UltroScan XL, LKB Bromma Co., California, USA).

II.5 Criteria used in interpreting results

A clinical sample was considered positive if a band of the appropriate size was visualized on an ethidium-stained agarose gel and this band hybridized specifically with the CMV probe following Southern transfer. A clinical sample was considered negative if no signal was observed following electrophoresis or hybridization, and β-actin DNA sequences were detected in the same samples. The result was considered indeterminate if β-actin DNA sequences were not detected. An assay was considered valid if the reaction mixture control and negative cell lysate control were negative and the positive control (CMV strain AD169) yielded detectable signal. If these conditions were not fulfilled, test sample results were not interpreted and the PCR reaction was repeated.

III. Results

III.1 Optimization of CMV PCR

Two critical parameters in the development of a new PCR protocol ($MgCl_2$ and primer concentrations) were evaluated in order to optimize the assay. This was done individually for each primer pair.

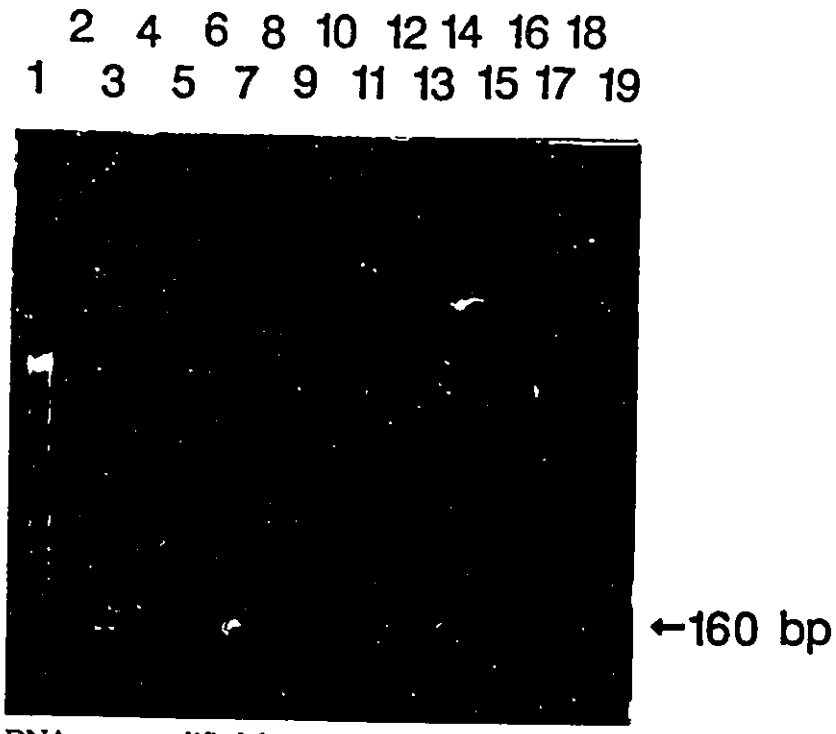
III.1.1 Primer Concentration

The primer concentrations were first titrated (0.3 - 1.5 μM for IE primers; 0.25 - 1.2 μM for LA primers). The primer concentrations were carefully chosen from previous publications on the two different primer sets (Cassol et al, 1989; Shibata et al, 1988). The selected concentration of primers for our further work was 0.4 μM for IE primers (Fig. 2, lanes 5 and 6) and 1.2 μM for LA primers (Fig. 4a lanes 3 and 4). With these concentrations, positive standard DNA from AD169 at 1:10 dilution in distilled water (dH_20) could be detected as a strong band on the gel electrophoresis. When IE and LA primers were used in higher concentrations (Fig. 2, lanes 8 - 19; Fig. 4A, lanes 6 - 11), extensive non-specific amplification was observed.

III.1.2 Magnesium ion concentration

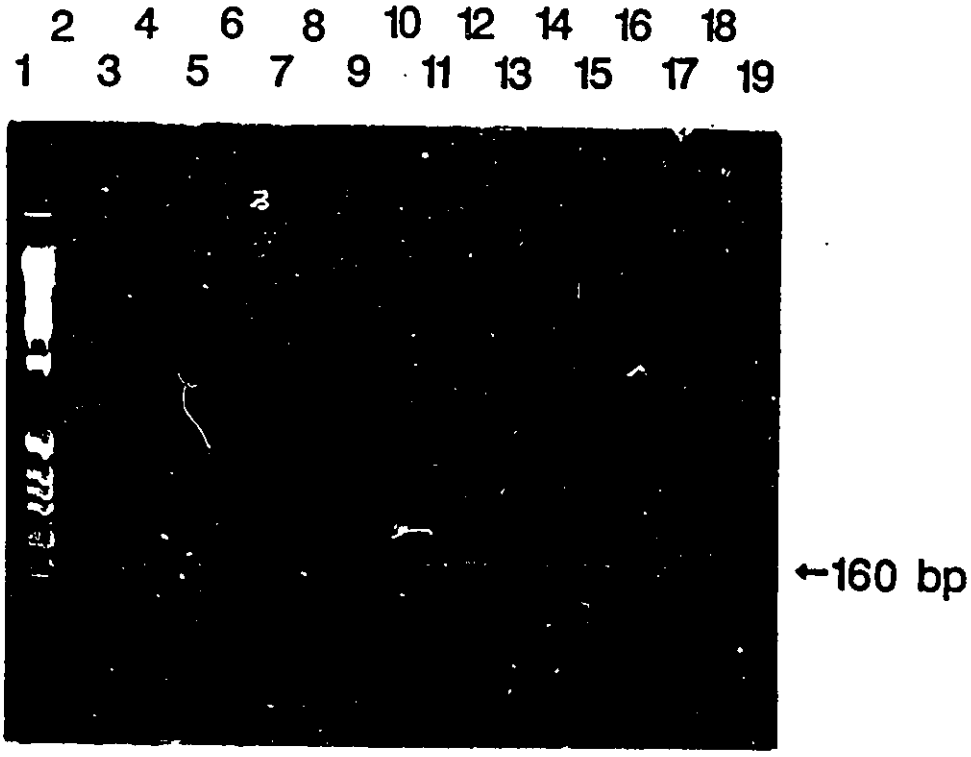
IE primers at 0.4 μM were tested with 0.5 - 2.5 mM $MgCl_2$ (Fig. 3, lanes 2 - 19). $MgCl_2$ concentration of 1.5 mM was optimum for reactions utilizing IE primers (Fig. 3, lanes 8 and 9). Non-specific amplifications were noted when the reaction mixture contained higher or lower $MgCl_2$ concentrations. In the LA primer system, $MgCl_2$ concentration was simultaneously titrated with the two primer concentrations (Fig. 4A and B).

Fig. 2: PCR was performed on CMV-positive cultures with different IE primer concentrations as outlined in Materials and Methods



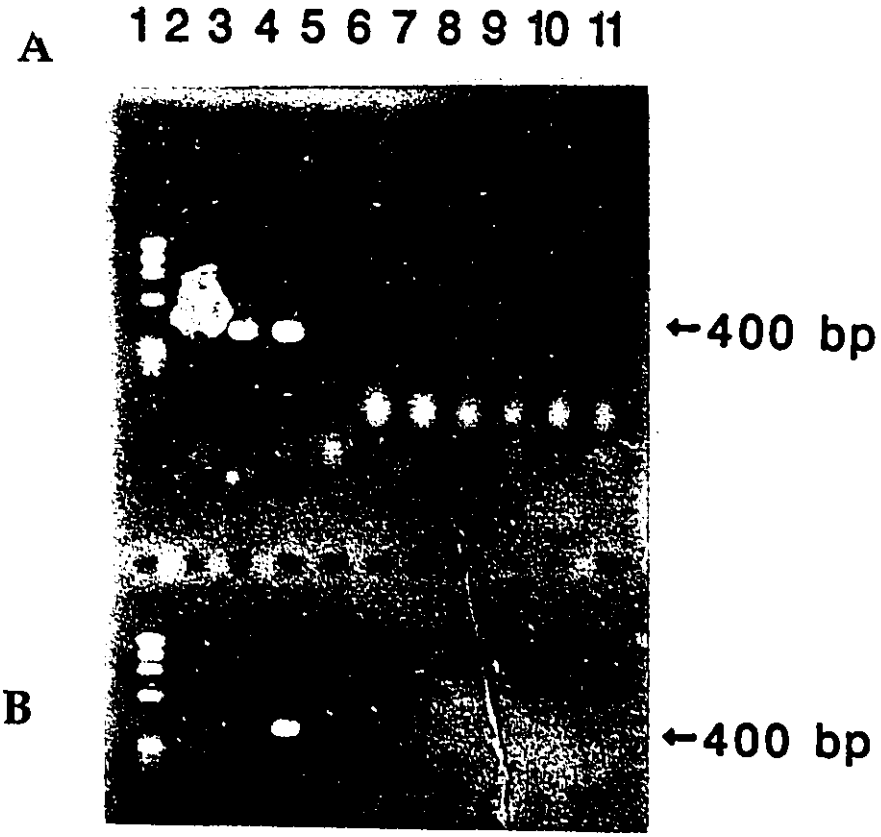
Legend: AD169 DNA was amplified for 39 cycles. Lane: 1, standard DNA marker - f174RF DNA/HaeIII. The following IE primer concentrations were used in each lane; 2 and 3, 0.3 µM; 5 and 6, 0.4 µM; 8 and 9, 0.5 µM; 11 and 12, 0.6 µM; 14 and 15, 1 µM; 17 and 18, 1.5 µM. The following reaction mixtures with different IE primers concentrations were used as negative controls; 4, 0.3 µM; 7, 0.4 µM ; 10, 0.5 µM; 13, 0.6 µM; 16, 1 µM and 19, 1.5 µM.

Fig. 3: $MgCl_2$ titration for CMV IE PCR.



Legend: AD169 (positive standard) was amplified for 39 cycles using IE primer pair at 0.4 μM Lane: 1, standard DNA marker, f174RF DNA/HaeIII. The following $MgCl_2$ concentrations were used in each lane; 2 and 3, 0.5 mM; 0.5 mM; 5 and 6, 1.0 mM ; 1.0 mM ; 8 and 9, 1.5 mM ; 1.5 mM ; 11 and 12, 1.75 mM ; 1.75 ; 14 and 15, 2.0 mM; 2.0 mM ; 17 and 18, 2.5 mM ; 2.5 mM. The following reaction mixtures with different $MgCl_2$ concentrations were used as negative controls in each lane; 4, 0.5 mM; 7, 1.0 mM; 10, 1.5 mM; 13, 1.75mM; 16, 2.0 mM and 19, 2.5 mM.

Fig. 4: $MgCl_2$ titration for CMV LA PCR.



Legend: The experiment was performed as described. AD169 in part A was amplified for 35 cycles with 1.2 µM LA primers; part B, 0.25 x µM. For part A and B, lane 1, f174RF DNA/HaeIII; the following $MgCl_2$ concentrations were used in each lane: 2, 0.5 mM; 3, 1.0 mM; 4, 1.5 mM; 5, 2.0 mM; 6, 2.5 mM; 7, 3.0 mM; 8, 3.5 mM; 9, 4.0 mM; 10, 4.5 mM; 11, 5.0 mM.

Thus, we evaluated 0.5 - 4.5 mM $MgCl_2$ (Fig. 4A and B, lanes 2 to 11). It was found that specifically amplified product was only detected at both primer concentrations in the presence of 1.5 mM $MgCl_2$ (Fig. 4A, lane 4; Fig. 4B, lane 4). This concentration was used in the remainder of our experiments.

III.2 Sensitivity and specificity of CMV PCR testing

To estimate the sensitivity achievable of our CMV PCR amplification protocol, AD169 DNA was serially diluted in seronegative PBMCs, seronegative PMNLs, distilled water or human placental DNA, then amplified using IE or LA primers. To assess the specificity of the system, the following samples were also amplified using both pairs of primers: bacterial DNA from *Neisseria gonorrhoeae* , human DNA from PBMCs, PMNLs and placenta and viral DNA from HSV-I, HSV-II and EBV.

Using the IE primer pair, specific signal was detected from 5 µl AD 169 suspension diluted in up to 10^5 PBMCs (Fig. 5, lane A7). Signal was also present using a 1:100 dilution of AD 169 in dH_2O as a substrate for amplification (Fig. 5, lane B5). Dilution of 5 µl AD 169 suspension in as few as 10^4 PMNLs (Fig. 5, lane A11) or any amount of human placental DNA (Fig. 5, lane B10), abolished any detectable specific signal.

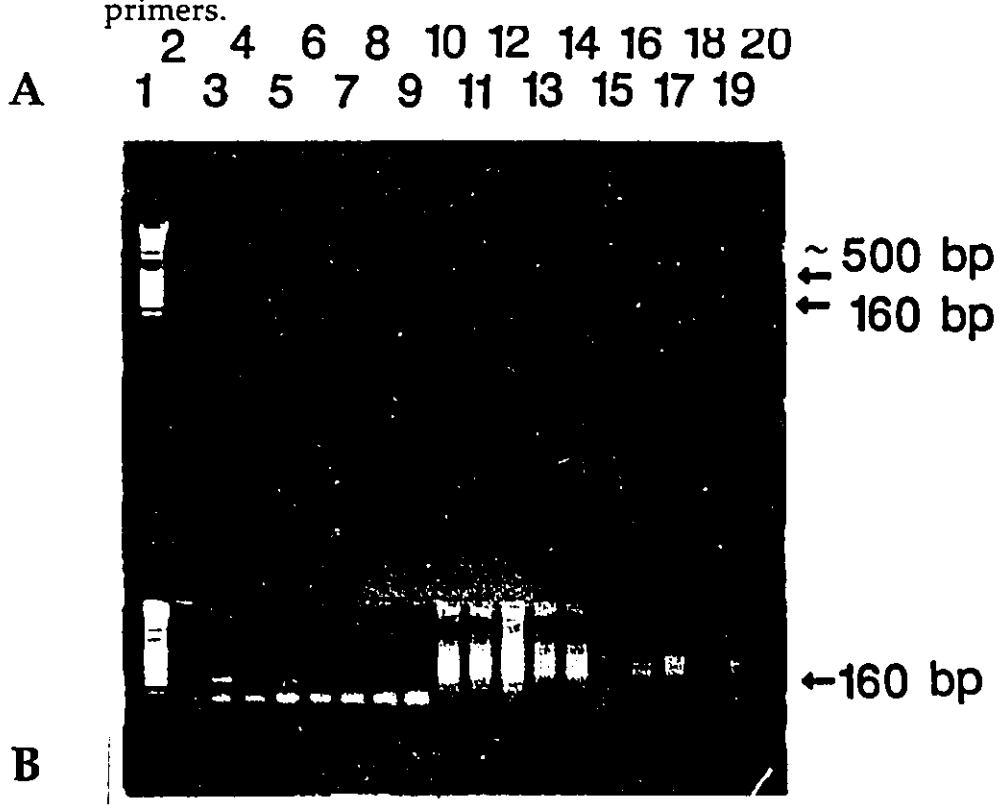
In assessing the specificity of the IE primer-based protocol, we noted extensive background bands on the gel when human placental DNA (Fig. 5, lanes B10-14) and viral DNA (Fig. 5, lanes B17-19) were used as substrates. Interestingly, a single band of approximately 500 bp in size was observed when *Neisseria gonorrhoeae* DNA was amplified (Fig. 5, lane A19).

Using the LA primer pair, no specific signal was detected from an undiluted AD 169 stock (Fig. 6, panel A), making the interpretation of data generated following dilution of this stock in seronegative PBMCs or PMNLs quite difficult. These experiments would have to be repeated. However,

dilution of a fresh stock with dH_2O (1:1000) (Fig. 6, lane B8) or human placental DNA (1:1000) (Fig. 6, lane B15) yielded a single, specific band of the appropriate size. No bands or background were detected following amplification of human placental DNA or other viral DNA alone (Fig. 6, lanes B16-19). Experiments with bacterial DNA were inconclusive.

Retesting three replicates of the same samples gave identical results. Based on the higher observed sensitivity of the LA primer pair (serial dilutions in dH_2O or human placental DNA) and the absence of non-specific amplification in this system, all further experiments were conducted using this primer pair.

Fig. 5: Sensitivity and Specificity of the CMV PCR assay using IE primers.

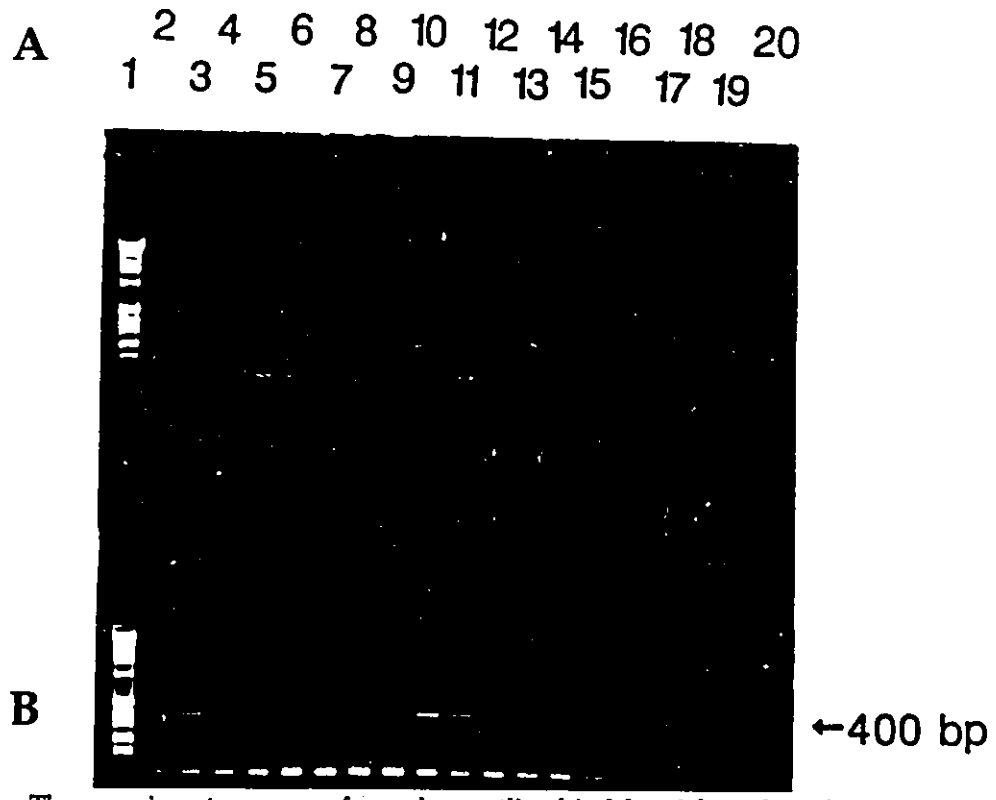


Legend: The experiments were performed as outlined in Materials and Methods. DNA from CMV, other herpesviruses, bacteria and circulating leukocytes were subjected to 39 cycles of amplification using the IE primer set. The positive control (AD169) was serially diluted as indicated in the text. Lanes: A1, standard DNA marker, f174RF DNA/HaeIII; A2, undiluted AD169; AD169 (5 µl) diluted in : A3, 10^4 PBMCs; A4, 2 x 10^4 PBMCs; A5, 5 x 10^4 PBMCs; A6, 7 x 10^4 PBMCs; A7, 10^5 PBMCs; A8, 1.5 x 10^5 PBMCs; A9, 2 x 10^5 PBMCs; A10,10^5 PBMCs alone; A11, 10^4 PMNLs; A12, 2 x 10^4 PMNLs; A13, 5 x 10^4 PMNLs; A14, 7 x 10^4 PMNLs; A15, 10^5 PMNLs; A16, 1.5 x 10^5 PMNLs; A17, 2 x 10^5 PMNLs; A18, PMNLs alone; A19, bacterial DNA alone; A20, reaction mixture alone.

Lanes: B1, standard DNA marker; B2, AD169; AD169 (5 µl) diluted in :B3, 1:10 dH_2O; B4, 1:50 dH_2O; B5, 1:100 dH_2O; B6, 1:200 dH_2O; B7, 1:500 dH_2O; B8, 1:1000 dH_2O; B9, dH_2O alone; B10, 1:10 hpDNA; B11, 1:50 hpDNA; B12, 1:100 hpDNA; B13, 1:200 hpDNA ; B14, 1:500 hpDNA; B15, 1:1000 hpDNA; B16, hpDNA alone; B17, HSV-I alone; B18, HSV-II alone , B19, EBV alone; B20, reaction mixture alone.

Abbreviations: PBMCs,Peripheral blood mononuclear cells; PMNLs, Polymorphonuclear leukocytes; dH_2O, distilled water; hpDNA, human placenta DNA; HSV-I and HSV-II, herpes simplex virus I and II; EBV, Epstein-Barr virus.

Fig. 6: Sensitivity and specificity of the CMV PCR assay using LA primers.



Legend: The experiments were performed as outlined in Materials and Methods. DNA from CMV, other herpesviruses, bacteria and circulating leukocytes were subjected to 35 cycles of amplification using the LA primer set. The positive control (AD169) was serially diluted as indicated in the text. Lanes: A1, standard DNA marker, f174RF DNA/HaeIII; A2, AD169; AD169 (5 µl) diluted in : A3, 10^4 PBMCs; A4, 2 x 10^4 PBMCs; A5, 5 x 10^4 PBMCs; A6, 7 x 10^4 PBMCs; A7, 10^5 PBMCs; A8,1.5 x 10^5 PBMCs; A9, 2 x 10^5 PBMCs A10, PBMCs alone; A11, 10^4 PMNLs; A12,2 x 10^4 PMNLs; A13, 5 x 10^4 PMNLs; A14, 7 x 10^4 PMNLs; A15, 10^5 PMNLs; A16, 1.5 x 10^5 PMNLs; A17, 2 x 10^5 PMNI s; A18, PMNLs alone; A19, bacterial DNA alone; A20, reaction mixture alone. Lanes: B1, standard DNA marker; B2, AD169; AD169 (5 µl) was diluted in B3, 1:10 dH_2O; B4, 1:50 dH_2O; B5, 1:100 dH_2O; B6, 1:200 dH_2O; B7, 1:500 dH_2O; B8, 1:1000 dH_2O; B9, dH_2O alone; B10, 1:10 hpDNA; B11, 1:50 hpDNA; B12, 1:100 hpDNA; B13, 1:200 hpDNA; B14, 1:500 hpDNA; B15, 1:1000 hpDNA; B16, hpDNA alone; B17, HSV-I alone; B18, HSV-II alone, B19, EBV alone; B20, reaction mixture alone.

Abbreviations: PBMCs, Peripheral blood mononuclear cells; PMNLs, Polymorphonuclear leukocytes; dH_2O, distilled water; hpDNA, human placenta DNA; HSV-I and HSV-II, herpes simplex virus I and II; EBV, Epstein-Barr virus.

III.3 Clinical CMV PCR

A total of 40 specimens were analyzed by CMV PCR: 35 from 25 HIV-seropositive individuals and five from healthy HIV-seronegative blood donors. Only PCR was performed on PBMCs as these cells are known to harbor latent rather than infectious virus (Ho M., 1991). Virus isolation and PCR were done on all isolated PMNLs.

Of the 35 PBMC samples collected from HIV-seropositive individuals, 10 were positive by PCR, 25 were negative. Among the five samples from HIV seronegative donors, one was positive by PCR (Table 2).

PMNL-based culture and PCR results were compared (Table 3). Of 35 PMNL samples from HIV seropositive individuals, one was positive by PCR and virus isolation, eight positive by PCR alone and 26 negative by culture and PCR. DNA from the five healthy HIV seronegative controls gave consistently negative results, by culture and PCR of PMNLs.

To determine the effect of the number of cells used in the amplification reaction mixture, 10 000 and 100 000 PBMCs and PMNLs of four HIV seropositive individuals were amplified in parallel (Fig. 7 and 8). Specimens containing 10 000 cells gave a weaker positive signal (Fig. 7) than that generated from 100 000 cells taken from the same patients at the same point in time (Fig. 8). Interestingly, specific signal was detected (Fig. 9, control PBMCs) in one of the HIV seronegative samples when 100 000 cells were used, indicating latent viral infection that was missed when a lower cell number was used as a target for amplification. From these results, we selected a standard of a 100 000 cell inoculum for our ongoing work. All positive PCR results are illustrated in figures 8 and 9.

Table 2: Detection of CMV in PBMCs of HIV seropositive (n=35) and seronegative (n= 5) samples using DNA PCR

	Patient Numbers (n)	Number of Samples (n)	PCR Positive	PCR Negative
HIV Seropositive	25	35	10	25
HIV Seronegative	5	5	1	4

Table 3: Detection of CMV in PMNLs of HIV seropositive (n=35) samples using DNA PCR and cell culture.

	Number of PCR results	
Culture results	Positive	Negative
Positive	1	0
Negative	8	26

Densitometer readings from amplified PBMCs (Fig. 8 and 9) were notably higher than those obtained from corresponding PMNLs. Only one patient (Fig. 9, 025) had a significantly higher reading in PMNLs than in PBMCs.

II.3.1 Correlation of CMV PCR testing of specimens with clinical disease.

The results of PCR testing, virus isolation and clinical status are summarized in Table 4. Most patients (7/9) with detectable CMV DNA in their PMNLs had very advanced immune disease (CD4 $\leq 10/mm^3$). Six patients had CMV retinitis at the time of sampling. Only one had a positive culture result. Five patients were subsequently treated for their disease and on follow-up became PCR-negative. The only patient with a positive culture also had a negative culture in follow-up. The patients with negative CMV PCR results did not have any evidence of clinical CMV disease.

Seven patients (012, 016, 017, 021, 023, 024 and 025, including all three with no evidence of CMV retinitis and positive PMNL-based PCR results) were evaluated for other evidence of CMV infection. One of the patients (016) was shown to have CMV pneumonitis. The other patients (012, 017, 021, 023, 024 and 025) had a febrile illness consistent with active CMV infection, that otherwise remained undiagnosed. The one (021) patient with positive PBMC-based PCR results and no evidence of CMV DNA in their PMNLs had no evidence of clinical CMV disease.

Fig. 7: DNA PCR results (10,000 cells)

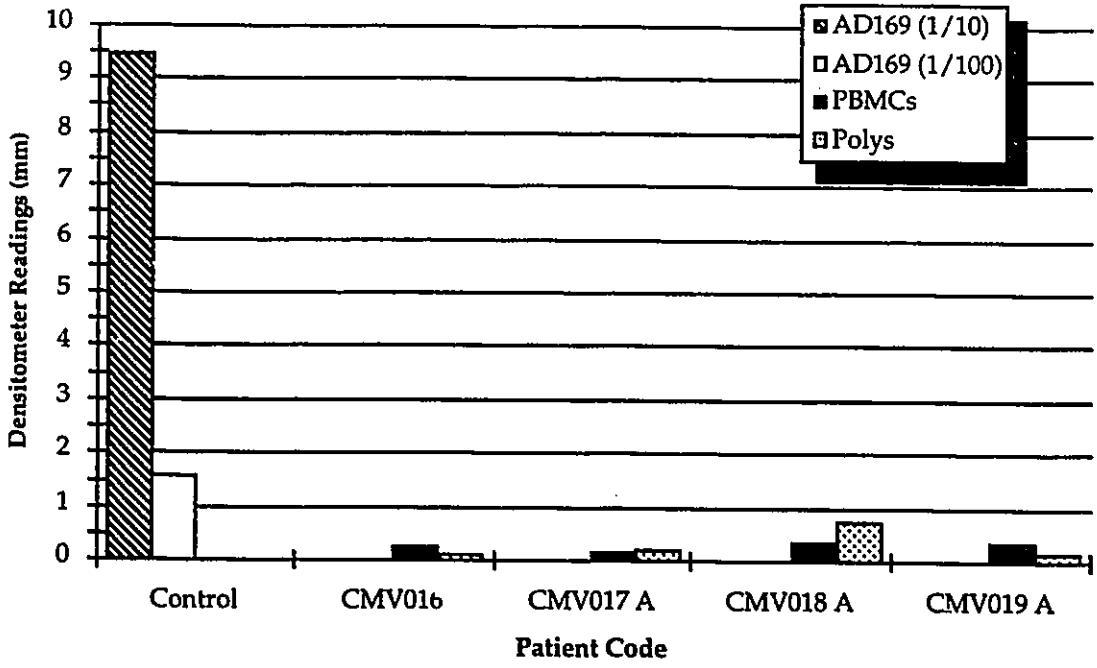


Fig. 8: DNA PCR results (100,000 cells)

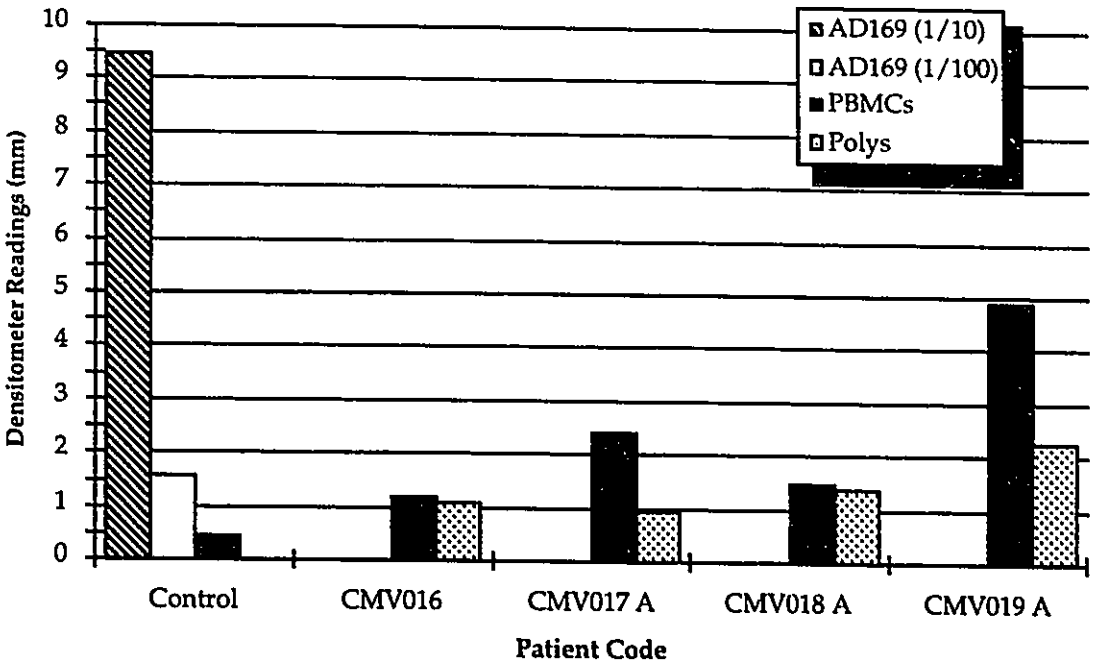
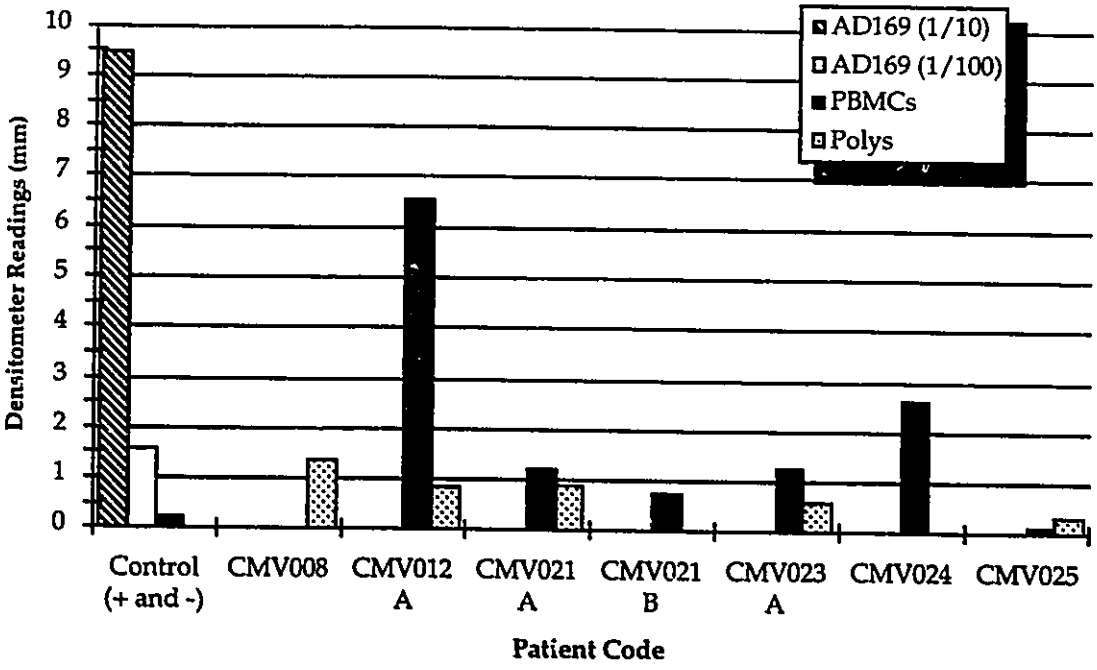


Fig. 9: DNA PCR results (100,000 cells)



III.3.2 Overall comparison of gel electrophoresis and Southern blot hybridization in detecting amplified products

The use of gel electrophoresis and/or Southern blot hybridization to detect amplified products was evaluated. Five PMNL preparations and six PBMC preparations were positive by direct gel electrophoresis with LA primers (Table 5). Southern blot hybridization could detect four more PMNL and three PBMC positive results previously undetected by gel electrophoresis. A comparison of direct gel and Southern blot analysis showed the combination of these two amplified product detection assays was able to detect CMV in PBMCs of 10 and PMNLs of nine samples taken from the HIV-seropositive individuals included in our study. Using the LA primer set and direct gel analysis to detect the amplified DNA product was significantly higher in sensitivity compared with cell culture. Use of Southern blot hybridization increased the overall sensitivity while preserving the specificity and predictive value of a positive test. Thus, we reported the Southern blot hybridization results in our work.

Tabie 4: Virologic and clinical summary of patients with full - blown AIDS whose PMNLs were evaluated sequentially by culture and PCR[a]

Patient[a]	Date	$CD4^+$ / mm^3	CMV Retinitis	R_X	Cell Culture	CMV PCR
008		83	Yes	0	–	+
012A	Sept./93	46	No	ACV	–	+
012B	Nov./93					–
016		<10	No	0	–	+
017A	July/93	<10	Yes	GCV	–	+
017B	Sept./93			GCV	–	–
018A	Nov.8/93	<10	Yes	0	–	+
018B	Nov.15/93			GCV,FCT	–	–
019A	July/93	<10	Yes	0	+	+
019B	Aug./93			GCV	–	–
019C	Nov./93			GCV	–	–
021A	Oct./93	<10	Yes	GCV	–	+
021B	Nov./93			GCV	–	–
023A	Sept./93	<10	Yes	GCV	–	+
023B	Oct./93			GCV	–	–
025		<10	No	0	–	+

a – A, B, or C represent multiple specimens of the same patient taken at different times

Abbreviations: R_X, treatment; 0 (R_X), no specific anti-CMV therapy; GCV, ganciclovir; ACV, acyclovir; FCT, foscarnet.

Table 5: Summary of discrepancies between PCR results when direct gel alone and direct gel with Southern blot hybridization were used to detect amplified products.

Patient[a]	PCR Results			
	PBMCs		PMNLs	
	Gel	SB[b]	Gel	SB[b]
008	–	–	+	+
012A	+	+	–	+
012B	–	–	–	–
016	+	+	–	+
017A	+	+	+	+
017B	–	–	–	–
018A	+	+	+	+
018B	–	–	–	–
019A	+	+	+	+
019B	–	–	–	–
019C	–	–	–	–
021A	+	+	+	+
021B	–	+	–	–
023A	–	+	–	+
023B	–	–	–	–
024	+	+	–	–
025	–	+	–	+

a – A, B, or C represent multiple specimens of the same patient

b – SB, Southern blot hybridization using LA probe to detect PCR product amplified by LA primers

IV. Discussion

There is an urgent need for rapid, sensitive, reproducible and quantifiable methods for the detection of CMV which can be used to identify active, impending or recurrent disease. The application of PCR for diagnosis has been hampered by the frequently positive signals obtained from PBMCs or other specimens without apparent correlation with CMV disease (Gerna et al, 1991; Spector, 1990, van der Bij et al, 1988). In this report, we have shown that CMV DNA could be detected in PMNLs of patients with AIDS and active CMV disease. Viral DNA was not detectable in PMNLs of individuals without CMV disease. Moreover, viral DNA was equally undetectable in PMNLs of patients on appropriate treatment or prophylaxis. This included follow-up samples obtained from patients in whom prior PMNL preparations had yielded a positive signal.

IV.1 Specificity and Sensitivity of the assay

The LA primers used in our PCR protocols were specific for CMV, with no detectable amplification signal following evaluation of human genomic DNA or a wide range of viral pathogens and bacteria. In contrast, the IE primer set could non-specifically amplified DNA from *Neisseria gonorrhoeae* in one evaluation. This may relate to sequence homology between the bacterial genome and CMV genes expressed in early infection. Although we did not confirm this by formal sequencing, it is worrisome in itself and would limit our confidence in this primer pair in more widespread application. The lower bands observed in Fig. 5A and 5B may be indicative of primer dimers.

PCR has previously been used to detect CMV DNA in leukocytes (Dihella et al, 1988; Shibata et al, 1988). Some of these assays have increased

their sensitivity by using two single PCR systems (so called "nested" amplification) and by detection of the amplimers by blotting and hybridization with radiolabelled probes. In our work, it was possible to detect a laboratory strain of CMV (AD 169) as well as a range of clinical CMV isolates with a high degree of sensitivity using a single round of amplification. The LA oligomers are complementary to a highly invariant gene coding for a structural glycoprotein present in early and in late infection (Pande et al, 1984; Ruger et al, 1987). When the LA primer set was used for CMV PCR detection we found DNA sequences in 10 PBMC and 9 PMNL preparations. It is quite possible that some clinical CMV strains from specimens with negative results lack the sequences in their late genes to allow detectable amplification in this system. If these non-amplified "alleles" sort independently, a low false negative rate would be expected. Further studies are necessary to test this hypothesis. Alternatively, additional technical experiments may allow us to improve the sensitivity of the assay we have designed and detect additional samples. This being said, it does not appear that any individuals with clinical evidence of CMV were missed in our PMNL-based testing (see below), suggesting that we may be at (or near) optimal "clinical" sensitivity.

PCR amplification in combination with oligonucleotide probe hybridization has several advantages over tissue culture techniques. The amplification step results in a significant increase in the sensitivity of viral DNA detection. Further, our own protocol permits the electrophoretic separation and accurate identification of the amplified viral sequences, even in crude preparations containing large amounts of cellular debris.

Our inability to detect CMV DNA diluted in seronegative PBMC or PMNL DNA using the LA primer pair is problematic. Given that the positive control in these experiments was negative, no firm conclusions can be drawn

from these data. We are reassured that this is a chance phenomenon, based on our further experiments showing excellent detection of CMV DNA diluted 1:1000 in human placental DNA. However, we have noted that dilution of AD169 in different background DNA preparations yields different amounts of PCR product, as determined by the intensity of hybridization. More accurate measurements of absolute CMV DNA may be obtained by end point dilutions of positive standards in human genomic DNA, or by the use of internal controls. Further experiments will be undertaken in this regard. The lesser sensitivity of cell culture compared with DNA amplification techniques for CMV detection in blood has been previously noted (Storch et al, 1994; Spector et al, 1992; Shibata et al, 1988; Cassol et al, 1989) and may reflect limitations of culture techniques. Since the PCR can amplify CMV from both infective and non-infective DNA, the assay was also more sensitive in our hands, as would have been expected.

IV.2 Clinical application

Although infection with CMV is extremely common, serious disease due to this virus is uncommon and appears to be limited to severely immunocompromised patients including those with advanced AIDS (Schooley, R.T., 1990; Bloom and Palestine, 1988). Disease in these persons is felt to result from reactivation of a latent infection or from a superinfection with a different strain as cell-mediated immunity wanes (Pasternack et al, 1990). This study supports the importance of cell mediated immunity in controlling CMV infection. The risk for developing CMV retinitis increased as the $CD4^+$ lymphocyte count fell. The risk was highest for those with counts of $<10/mm^3$. In five of the six patients diagnosed with CMV retinitis the most recent count was $<10/mm^3$.

Depending on geographic location and socioeconomic conditions, seroprevalence rates for CMV vary from 40% to 80% (Ho, M., 1991). Possibly, given sufficient time, most patients with HIV potentially could develop CMV disease such as retinitis. Based on this, screening to detect early or asymptomatic CMV retinitis is indicated. Screening, however, implies that early treatment of CMV disease is of benefit, a hypothesis that may be correct in HIV seropositive individuals (Bailey et al, 1993). Current recommendations include screening patients with AIDS and $CD4^+$ lymphocyte counts $<100/mm^3$ every two to three months (Henderly et al, 1991). This study however suggested that CMV retinitis was most likely to develop when $CD4^+$ lymphocyte counts reached $<10/mm^3$. It appears that as persons infected with HIV continue to live longer, our reliance on more sensitive techniques in guiding treatment, prophylactic regimens and screening programs will only increase (Crowe et al, 1991).

The data presented here suggest that PCR could be helpful in predicting which HIV-infected patients are at risk for developing clinically relevant CMV disease, such as CMV retinitis. Most patients will have had a remote primary CMV infection when diagnosed, which will reactivate in the setting of advanced immune disease.

Patients infected with HIV did have CMV DNA detected in their PBMCs (10 out of 35) and PMNLs (9 out of 35). Our specific application of PCR to PMNLs was meant to address the issue of the inability of this technique to differentiate infectious from non-infectious virus. In theory, no latent CMV non-infectious virions are present in PMNLs. The amplification signals in PMNLs were generally weaker compared to PBMCs, as would be expected, due to the higher load of non-replicating virions in these cells. Stronger CMV amplification signals were obtained from the blood specimens of a few

immunocompromised patients (all with AIDS, some with recently diagnosed CMV retinitis). Our ability to detect DNA sequences in 8 culture-negative PMNL preparations suggests that we have in fact, developed a more sensitive assay for the detection of infectious CMV. In this regard, it is reassuring that the PCR assay did not detect CMV DNA from PMNLs of healthy HIV-seronegative volunteers, a result similar to previously published studies (Hsia et al., 1989; Olive at al., 1989; Storch et al., 1994).

There may be a role for serial CMV DNA PCR to be performed in high risk patients. Some immunocompromised patients were consistently positive for CMV DNA in PBMCs and negative in PMNLs, most of whom had $CD4^+$ lymphocyte counts of $<100/mm^3$ at the time of sampling. Six patients with AIDS and CMV retinitis maintained CMV DNA in their PMNLs at the time of evaluation. Some of these patients were previously receiving antiviral therapy. This persistence of CMV viremia despite CMV therapy has previously been described (Henderly and Jampol, 1991) and may reflect the severity of the immunodeficiency, decreasing the efficacy of therapy. The nine patients with positive PCR results in PMNLs showed a striking correlation between clinical immunodeficiency and CMV disease. Longer term studies will be necessary to elucidate if the presence of CMV DNA in PMNLs predicts clinical outcome. It is interesting to note that, in five patients on appropriate therapy, PCR signals became negative over time.

There are several possibilities to explain the lack of CMV DNA in PMNLs of four AIDS patients with CD4 counts of $<10/mm^3$. One possibility is that the PCR assay is not sufficiently sensitive to detect the very low levels of virus present in these persons. This possibility could, in theory, be resolved by further optimizing our PCR protocol. Alternatively, it could indicate that these individuals harbor strains of CMV that are not detectable

by the present PCR assay or that they carry viral genomes that are defective in the LA region. This could be addressed by repeating PCR using primers complimentary to a highly conserved (but unrelated) gene segment. A third possibility is that these individuals are not productively infected with CMV at the time of sampling. In light of our findings, such patients should be followed up periodically to address these issues. This will be done within the context of a prospective cohort study, to be planned in our center.

Even acknowledging some of these potential limitations, PCR appears to have potentially greater sensitivity than the currently used standard for CMV diagnosis (tissue culture) in predicting CMV disease. In summary, our findings indicate that CMV DNA can be detected in PMNLs of patients with AIDS at risk of CMV disease and that there is an excellent correlation between PCR positive results in PMNLs and CMV clinical disease. This powerful tool promises to be a useful procedure for monitoring patients suspected of having impending, acute or recurrent CMV disease, and its specific role in clinical medicine will be resolved by further study.

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