Relationship Between Haemorheological Factors and Cytomegalovirus Load in the Blood with Cytomegalovirus Retinitis in Patients with the Acquired Immune Deficiency Syndrome.

By Adnan Tufail for the degree of Doctor of Medicine (M.D.) in the University of London.

Jules Stein Eye Institute, University of California Los Angeles
Institute of Ophthalmology, University of London
Abstract

The purpose of the studies reported in this thesis is to evaluate factors that may help determine risk of developing active cytomegalovirus (CMV) retinitis in patients with human immunodeficiency virus infection. The studies were grouped into three broad categories, CMV load in the peripheral blood, haemorheologic measures in the peripheral blood, and measures of retinal capillary blood flow.

Quantitative CMV load was studied using a hybrid capture system. The hybrid capture assay was initially compared to culture techniques and qualitative polymerase chain reaction (PCR) methods of assessing CMV, and was found to be more sensitive than culture techniques but less sensitive than the qualitative PCR assay used. A second study, an observational case series, followed two cohorts of HIV-positive and CMV antibody positive patients (10 without CMV disease, and 11 with CMV retinitis). Elevated or rising CMV DNA blood levels appeared to be associated with the development of CMV disease in patients with low CD4+ T-lymphocyte counts. In patients with CMV retinitis, rising levels appear to be associated with the development of extraocular CMV disease or reactivation of retinitis. Conversely reactivation of retinitis occurred in the absence of changes in CMV DNA blood levels.

A number of haemorheologic factors that affect blood viscosity, including polymorphonuclear cell rigidity were assessed in HIV-infected patients with and without CMV retinitis. A significant increase in polymorphonuclear rigidity and lower haematocrit was found in patients with CMV retinitis. A correlation of red cell aggregation and zetacrit sedimentation ratio was found with CD4+ T-lymphocyte
count. No significant differences were found in retinal capillary blood flow, as measured by scanning Laser Doppler Flowmetry, between HIV-positive patients with and without CMV retinitis.

We have postulated that there is an interaction between virus load and retinal capillary blood flow that determines the risk of developing CMV retinitis.
CONTENTS

List of Figures

List of Tables

List of Abbreviations

Acknowledgements

Chapter 1 Aim and Scope of the Thesis

Chapter 2 Introduction and Background

2.1 Cytomegalovirus Retinitis

2.2 Historical Background

2.3 Virology

2.4 Epidemiology

2.4.1 Transmission

2.4.2 Immunosuppressed Patients

2.4.3 Ocular Disease

2.4.4 Cytomegalovirus Retinitis in Patients with AIDS.

2.5 Risk Factors

2.5.1 Ocular Disease

2.5.1.1 CMV Retinitis in Patients with AIDS

2.6 Clinical Features

2.6.1 Immunosuppressed Patients
2.6.2 Ocular Disease

2.7 Pathology

2.7.1 Ocular Disease

2.8 Pathogenesis

2.8.1 Spread of the Virus

2.8.2 Chronic Infection and Latency

2.8.3. Immune Response

2.8.3.1 Humoral Immunity

2.8.3.2 Cell-Mediated Immunity and Cytokines

2.8.4 Effect of Cytomegalovirus on Host Immunity

2.8.5 Factors Affecting Disease Severity

2.9 Treatment

2.9.1 Monitoring Therapy

2.9.2 Intravenous Therapy

2.9.3 Intraocular Therapy

Chapter 3 Cytomegalovirus DNA Load Measurements

3.1 Introduction

3.2 Quantitative assays for cytomegalovirus

3.2.1 Quantitative viral cultures

3.2.2 Quantitative pp65 antigenaemia assay
3.2.3 Quantitative polymerase chain reaction assays

3.2.4 Branched-DNA signal amplification assay

3.2.5 Solution Hybridization DNA assay

3.3 Comparison of hybridisation, culture and PCR for CMV detection

3.3.1 Specimens

3.3.2 Culture.

3.3.2.1 Cell Culture

3.3.2.2 Shell Vial Assay

3.3.3 Hybrid capture system

3.3.4 Polymerase Chain Reaction

3.3.5 Results

3.3.6 Conclusions

3.4 Main Study: Quantitative CMV DNA levels in the blood and its relationship to CMV retinitis

3.5 Materials and Methods

3.5.1 Patients

3.5.2 Cytomegalovirus DNA Analysis

3.5.2.1 Method summary

3.5.3 Method details

3.5.3.1 Specimen Collection and Handling
3.5.3.2 Specimen Preparation
3.5.3.3 Standard and Control Preparation
3.5.3.4 Denaturation and Hybridization
3.5.3.5 Hybrid Capture
3.5.3.6 Hybrid Detection
3.5.3.7 Washing
3.5.3.8 Signal Generation
3.5.3.9 Quality Control
3.5.3.10 Interpretation of specimen results

3.5.4 Data Analysis
3.5.5 Results
3.5.6 Discussion

Chapter 4 Haemorheologic Abnormalities

4.0 Introduction

4.1 Principles of Clinical blood rheology

4.1.1 Introduction
4.1.2 Blood Rheology
4.1.3 Blood viscosity

4.1.3.1 Plasma viscosity
4.1.3.2 Haematocrit
4.1.3.3 Temperature

4.1.3.4 Shear rate

4.1.3.5 Red cell deformation

4.1.3.6 Red cell aggregation

4.1.4 Blood viscosity in narrow vessels

4.1.5 White blood cell rheology

4.1.6 Rheological properties of neutrophils in their active state

4.1.7 Flow of leukocytes through vessel branches

4.1.8 Adhesion molecules

4.1.9 Coagulation abnormalities

4.2 Measurable Rheologic Parameters

4.2.1 Erythrocyte Sedimentation Rate

4.2.2 Zeta Sedimentation Ratio

4.2.3 Plasma Viscosity

4.2.4 Immunoglobulins and Immune Complexes

4.2.5 Red cell aggregation

4.2.6 Summary of Global Measures of Aggregation

4.3 Clinical Studies

Study 1 Neutrophil Deformability

4.3.1 Materials and Methods
4.3.1.1 PMN Preparation

4.3.1.2 PMN deformability measurements

4.3.1.3 Statistical Analysis

4.3.3 Results

4.3.4 Discussion

Study 2 Other Rheologic Measures

4.4.1 Materials and Methods

4.4.1.1 Laboratory Studies

4.4.1.2 Myrenne Aggregometer System

4.4.1.3 Statistical Analysis

4.4.2 Results

4.4.3 Discussion

Chapter 5 Retinal Capillary Blood Flow

5.1 Aim

5.2 Introduction

5.2.1 Retinal blood flow

5.2.1.1 Regulation of retinal blood flow

5.2.1.2 Retinal blood flow in HIV-infected patients

5.2.2 Techniques of measuring retinal blood flow in humans

5.2.2.1 Blue Field Entoptic Simulation
5.2.2.2 Scanning Laser Angiography

5.2.2.3 Laser Doppler Flowmetry

5.2.3 Choice of Technique for this Study

5.3 Scanning Laser Doppler Flowmetry

5.4 Reliability of method

5.5 Repeatability and Validity in HIV-infected individuals

5.5.1 Repeatability

5.5.1.1 Results

5.5.1.2 Discussion

5.5.2 Validity

5.5.2.1 Results

5.6 Main Study: Measurement of Retinal Capillary Blood Flow in HIV-infected Individuals

5.6.1 Aim

5.6.2 Methods

5.6.2.1 Statistical Methods

5.6.3 Results

5.7 Limitations of the Method

Chapter 6 Conclusions

6.1. Quantitative CMV DNA load
6.1.1 Validation of the solution hybridization assay

6.1.2 Blood CMV DNA levels in patients without CMV disease at baseline

6.1.3 Blood CMV DNA in patients with CMV disease at baseline

6.1.4 Future studies utilising quantitative CMV DNA load

6.2. Blood Rheologic Factors

6.2.1 Abnormalities in rheologic factors associated with the HIV Disease

6.2.2 Future studies

6.3. Retinal Capillary blood flow

6.3.1 Validation of HRF

6.3.2 Abnormalities in retinal capillary flow associated with the CMV Disease

6.3.3 Potential and future applications of measuring retinal capillary blood flow

6.4 Concluding remarks

Chapter 7 References

Chapter 8 Appendix

8.1 Background to Laser Doppler Flowmetry

8.2 Presentations and publications arising from these studies
List of Figures

2.1 CMV retinitis – fulminant/oedematus
2.2 CMV retinitis – indolent granular
2.3 CMV retinitis – satellite border
2.4 CMV retinitis – treated/inactive
3.1 Relational probe map
3.2 Changes in CMV DNA blood levels over time in patients with no CMV disease at baseline
3.3 Boxplots showing distribution of CMV DNA blood levels in patients with CMV retinitis at baseline
3.4 Changes in CMV DNA blood levels over time in patients with clinically inactive CMV retinitis
3.5 Changes in CMV DNA blood levels over time in a patient, who had persistent clinical activity
3.6 Changes in CMV DNA blood levels over time in a patient, who had fluctuating clinical activity
4.1 Cotton-wool spots
4.2 CTA Diagram
4.3 Correlation between transit time and CD4+ T-lymphocyte count
5.1 SLDF example output colour coded by the flow, volume, or velocity
5.2a Relationship between flow measurement and alteration in target speed
5.2b Relationship between flow measurement and alteration of target focus

5.2c Relationship between flow measurement and alteration of target haematocrit

5.3 Boxplots showing distribution of retinal blood flow by region in patients with and without HIV infection

6.1 Proposed interaction of virologic and haemorheologic factors in the pathogenesis of CMV retinitis
List of Tables

3.1 Summary of studies of quantitative assays for CMV load in the blood
3.2 Comparison of cell culture and hybrid capture
3.3 Sensitivity-excluding PCR
3.4 Comparison of hybrid capture and qualitative PCR
3.5 Cutoff calculation
3.6 CMV Genome Equivalents Complementary DNA
3.7 Characteristics of patients without CMV retinitis at baseline
3.8 Characteristics of patients with CMV retinitis at baseline
4.1 CTA - Comparison of HIV-infected individuals with HIV-negative controls
4.2 CTA - Comparison of HIV-infected individuals with and without CMV retinitis
4.3 Other rheologic measures subject groups
5.1a The coefficients of variation for each of the non-HIV infected subjects
5.1b The coefficients of variation for each of the HIV infected subjects
5.2 Descriptive statistics of baseline comparisons between the groups
5.3 Capillary blood flow by region
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>BDNA</td>
<td>Branch deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate-conjugated</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HCS</td>
<td>Hybrid capture system</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HRF</td>
<td>Heidelberg Retina Flowmeter</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon-alpha</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Interferon-beta</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>LFA-3</td>
<td>Leukocyte function antigen-3</td>
</tr>
<tr>
<td>LDF</td>
<td>Laser Doppler Flowmetry</td>
</tr>
<tr>
<td>LDV</td>
<td>Laser Doppler velocimetry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>SLDF</td>
<td>Scanning Laser Doppler Flowmetry</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>SLDV</td>
<td>Scanning laser Doppler velocimetry</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>VAR</td>
<td>Variability</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
</tbody>
</table>
Acknowledgements

My interest in CMV retinitis started as an SHO at Moorfields Eye Hospital in the early to mid 1990s at a time when there was an increasing incidence of CMV retinitis being seen in London. Professor Lightman encouraged my interest and helped provide me with a unique opportunity to be able to study with Professor Gary N. Holland at the Jules Stein Eye Institute, Los Angeles, California.

Many colleagues gave me valuable assistance with the research, including Dr Marjorie Miller (for work relating to the hybrid capture assay, CMV PCR, and shell vial assay), and all the members of The Centre for Clinical AIDS Research and Education, UCLA School of Medicine, especially Dr Ardis Moe, for helping recruit patients and provide information regarding the general health of study patients. Professor Herbert Meiselman, and Dr Tim Fisher, from The Department of Physiology and Biophysics, University of Southern California School of Medicine, introduced me, with enthusiasm and humour, to the world of haemorheology and Ms. Rose Wenby patiently instructed me in the various haemorheologic assays. All of Professor Gary Holland’s staff (especially Ms Catherine Strong) gave me constant administrative support, which allowed for the regular review of study patients.

I would like to thank my parents and brother (who have always encouraged me in a quest for knowledge) and also my friends for bearing with me during the completion of this thesis.

The research was supported in part by grants obtained by Professor Gary N. Holland: these include a grant from the Elizabeth Taylor AIDS Foundation, Los
Angeles, Research to Prevent Blindness Inc., New York, and National Institutes of Health grants EY08057 and AI27660.

I could not have completed this thesis without the tremendous support and motivation of Professor Susan Lightman throughout the course of the research and writing up. My biggest debt of gratitude is to Professor Gary N. Holland, for taking me on, instructing and focusing my clinical and laboratory skills, for the huge effort and time that he put into my projects, and his unwavering enthusiasm support, motivation, patience, and friendship.
Chapter 1  Aim and Scope of the Thesis

New strategies are needed to identify individuals with human immunodeficiency virus (HIV) infection who are at risk for development of cytomegalovirus (CMV) end-organ disease, and to manage disease reactivations more effectively in those patients who already have CMV retinitis. Although prophylactic therapy exists, both cost and potential toxicity limits its use, especially in view of the fact that 60% to 75% of patients with AIDS may never develop CMV disease. (Holland et al. 1995; Hoover et al. 1996) For effective use of prophylaxis, there needs to be a better understanding of the groups at greatest risk, so that low-risk patients will not be treated unnecessarily. With the potential for longer patient survival associated with new combination antiretroviral therapies, it is increasingly important to limit reactivation of CMV retinitis, which can result in disease progression, with destruction of additional retinal tissue and loss of vision.

Several hypotheses exist that try to explain the high prevalence of CMV retinitis in HIV-infected individuals; the level of immunosuppression, allowing a high blood virus load and virus replication in tissue, and disruption of blood flow and the blood retinal barrier, that may effectively increase the chance of CMV entering retinal tissue. Animal murine CMV models support the notion that both factors may play a role, (Atherton et al. 1992; Duan et al. 1994; Duan et al. 1996), and the association of abnormal blood flow to CMV retinitis development is also supported by clinical observation. (Jabs, 1995)
With the introduction of a prophylactic therapy, oral ganciclovir, that has potential side effects and is expensive it would be useful to target only those patients at risk of developing CMV disease. We hypothesise that the development of CMV retinitis in AIDS patients is multifactorial and depends on a combination of increasing virus load and decreased blood flow due to alteration in blood constituents and vessel wall damage.

Little is currently known about the above factors in HIV-infected patients. The aim of this study is to evaluate several quantifiable risk factors associated with CMV retinitis, that may result in the development of predictive tests (allowing targeted use of prophylactic therapy to those most at risk of developing active retinitis) or may allow for alteration of risk factors to prevent the disease occurring.

A number of factors were studied and related to clinical measures or outcomes, the studies looked at three areas:

1) Cytomegalovirus DNA load in the peripheral blood with relation to the development or reactivation of CMV retinitis (chapter 3).

2) Haemoreologic abnormalities, notably leukocyte deformability as measured by a cell transit analyser (chapter 4).

3) In vivo alterations in retinal capillary blood flow measured Laser Doppler Flowmetry (chapter 5).

The evaluation of other risk factors, like the loss of specific CMV immunity were beyond the scope of this thesis.
Where necessary preliminary work was undertaken to evaluate a particular test, and these evaluations are grouped in the relevant chapter.
Chapter 2 Introduction and Background

2.1 Cytomegalovirus Retinitis

Cytomegalovirus (CMV) is a ubiquitous member of the herpes group of viruses. Infection with CMV is very common among the general population, but in most cases it does not cause clinically apparent disease. Cytomegalovirus is, however, a well-known cause of serious, even life-threatening, disease in immunosuppressed individuals, and in congenitally infected newborns. Cytomegalovirus retinitis has emerged from obscurity since the 1980's to become an important cause of blindness because of its association with the acquired immunodeficiency syndrome (AIDS); in fact, because of the AIDS epidemic, CMV retinitis is now believed to be the most common single cause of posterior uveitis in many large urban areas of North America.

2.2 Historical Background

The history of research into CMV and the diseases it causes has been outlined by Ho. (Ho, 1991) In 1921, Goodpasture and Talbot introduced the term "cytomegalia" to describe the presence of large mononuclear inclusions in various organs in the autopsy of a 6 week old child. (Goodpasture and Talbot, 1921) Similar findings in newborns suspected to have syphilis or other congenital infections had
been described earlier in the century by other investigators, who thought that the
cytomegalic cells were protozoa. Goodpasture and Talbot disagreed with this theory,
as did subsequent investigators. In 1925, von Glahn and Poppenheimer described
identical findings for the first time in an adult, and suggested that the inclusions were
caused by a virus similar to those of the herpes virus group. (Von Glahn and
Pappenheimer, 1925) Over the next several years, additional congenital cases were
reported in symptomatic newborns, and a viral causation became accepted.

In 1932, Farber and Wolbach showed in a large autopsy series that clinically
inapparent infection could also occur. (Farber and Wolbach, 1932) In 1950, Wyatt
and associates suggested the term "generalized cytomegalic inclusion disease" to
describe the lethal infection in newborns, and showed that the diagnosis could be
made on cytologic examination of the urine. (Wyatt et al. 1950)

The viral agent responsible for cytomegalic inclusion disease was first
visualized by electron microscopy in 1953 by Minders. (Minders, 1953) In the mid-
1950s, CMV was finally isolated and grown in tissue culture independently by
Smith, (Smith, 1954) by Weller and associates, (Weller et al. 1957) and by Rowe and
associates. (Rowe et al. 1956)

The spectrum of diseases known to be caused by CMV was expanded in 1965,
when Klemola and Kaariainen described a CMV-associated infectious
mononucleosis-like syndrome in immunocompetent adults. (Klemola and Kaariainen,
1965) More important in recent years has been the role of CMV as an opportunistic
pathogen in immunosuppressed patients; the growing number of bone marrow and
solid organ transplantations, and the increasing use of immunosuppressive drugs, was
associated with the emergence of pneumonitis, colitis, hepatitis, encephalitis, and other life-threatening diseases caused by CMV. (Ho et al. 1975) Then in 1981, CMV was recognized as one of the most common opportunistic infections in patients with AIDS.

CMV infection of the eye was first recognized in newborns with cytomegalic inclusion disease. In 1947, Kalfayan reported the presence of cytomegalic cells with nuclear inclusions at the "sclerocorneal junction," in episcleral tissue, and in ciliary body tissue of one infant. (Kalfayan, 1947) As more cases were seen in the 1950's and 1960's, it became obvious that the retina was the primary site of ocular disease in infants with disseminated CMV infection. (Dvorak-Theobald, 1959; Manschot and Daaman, 1962; Miklos and Orban, 1964; Smith, 1964; Tarkkanen et al. 1972)

The first confirmed case of CMV retinitis in an adult was reported by Smith in 1964; (Smith, 1964) a 61 year old woman who had been treated with chemotherapy for Hodgkin disease was found to have bilateral retinal necrosis with typical cytomegalic cells in the retina at autopsy. Moeller and associates (Moeller et al. 1982) were able to identify less than 50 reported cases in the English language literature of CMV retinitis in patients with non-congenital CMV disease before the AIDS epidemic. (Smith, 1964; Malek and Kisken, 1970; Aaberg et al. 1972; Cox et al. 1975; Augsburger and Henry, 1978; Meredith et al. 1979; Berger et al. 1979; Pollard et al. 1980).
2.3 Virology

Cytomegalovirus is an enveloped, linear, double-stranded DNA virus. It is a member of the family *herpesviridae* and subfamily *betaherpesvirinae*. Human CMV has been designated type species human herpes virus 5 (human cytomegalovirus) by the International Committee on Taxonomy of Viruses. (Matthews, 1979)

Cytomegalovirus is species-specific; in addition to human CMV, there are well-characterised CMV strains that affect the rat, mouse, monkey, and guinea pig. All CMV are similar to other herpes viruses in morphology and in their ability to produce latent infections and undergo reactivation.

Morphologically, CMV has a central DNA-containing core surrounded by a capsid composed of 162 capsomeres, each of which is hexagonal in cross section. The capsid is surrounded by a poorly demarcated area, the tegmentum, and an envelope. The genome of CMV is the largest of the herpes virus group. It is approximately 230,000 base pairs in length and has a molecular weight of 15,106. (Somogyi et al. 1986; Geelen et al. 1978) The DNA structure is similar to that of herpes simplex virus; they both contain long and short unique sequences bounded by terminally repetitive segments. The long and short sequences can be orientated in two directions so that four DNA isomers are produced by cells in culture. The genome has a coding capacity for over 200 proteins of average molecular weight. (Landini and Michelson, 1988) The entire CMV genome has been sequenced; (Bankier et al. 1991) it contains areas that are homologous with human chromosomal DNA, (Ruger et al. 1984) which has led to speculation that CMV may be oncogenic. The genome also contains an area of homology with HLA Class I
specificities (UL18 gene), that has been implicated in acting as a decoy for NK cells. (Hassan-Walker et al, 1998)

The cells in which CMV initially replicates and the sites of persistence have not been clearly defined. Thus, the nature of the virus receptor is not known. Cytomegalovirus replicates readily in fibroblasts in vitro, and much of our understanding of the virus was initially derived from such studies. Other human cell types are only moderately permissive in culture. (Knowles, 1976) Recently the importance of CMV behaviour in a particular cell type has been explored, including endothelial (Waldman et al. 1991; Vossen et al. 1996) and retinal pigment epithelial cells. (Maidji et al. 1998)

A cascade system is involved in controlling the synthesis of proteins from the genome of CMV. (Stinski et al. 1980) The immediate-early or α group of proteins are synthesized first, which allows the transcription of messenger RNA for the second group of proteins, the early or β proteins. Immediate-early proteins are rapidly expressed in the host nucleus after virus absorption and penetration; their presence may be the only evidence of infection in cells where the virus does not replicate. (Mocarski and Stinski, 1979) Early proteins allow DNA replication to proceed by encoding replicative enzymes such as DNA polymerase. The late or γ proteins are the last to be synthesized. Most or all of the immediate-early and early proteins are non-structural, while most of the late proteins are structural for the virus.

Clinical studies by Emery and co-workers suggest that CMV DNA replication in vivo is a highly dynamic process with a doubling time/half-life of CMV in the blood of approximately 1 day. (Emery et al. 1999)
Cytomegalovirus may cause abortive infection in some cells and lytic infections in others. In non-permissive cells, the lytic cycle can be blocked at any of the steps between absorption and virion production. The outcome of CMV infection is host cell-dependent and species-specific. Different CMV vary with respect to the range of host cells in which there is expression of genes for immediate-early proteins (which may have implications for understanding latency and abortive infections) and in which viral DNA replication can occur. (Lafemina and Hayward, 1988) Human CMV is the most restricted for both.

Infectious enveloped virus particles result when nucleocapsids, formed in the nucleus of the host cell, acquire an envelope that is derived from both the endoplasmic reticulum and the internal nuclear membrane of the cell. (Severi et al. 1979) The envelope glycoproteins, such as the gB and the gH complex contain antigens that stimulate host immune responses. (Marshall et al. 1994; Rasmussen et al. 1994; Alberola et al. 1998)

A number of different human CMV strains can be differentiated by both the size and number of additions to the terminal ends of the long and short components of the genome. (Stinski, 1991) The restriction endonuclease profiles of various human CMV strains share many similarities, but no two profiles are identical.

Strain variation may have implications in clinical drug resistance. Drug resistance in HCMV is conferred by mutations in the DNA polymerase gene (UL54) and/or the phosphotransferase gene.
2.4 Epidemiology

Cytomegalovirus infects a large proportion of adults throughout the world; rates vary depending on the group being studied, but in many populations it exceeds 50%. The prevalence of seropositivity increases with age;{Wentworth & Alexander 1971 ID: 283} it varies geographically, with higher rates found in developing countries,(Krech, 1973) and it is more prevalent in lower socio-economic groups.(Krech, 1973) Serological evidence of CMV infection, in the United States, is lowest among heterosexual patients with AIDS, but is almost universal among HIV-infected homosexual or bisexual men.(Drew et al. 1981)

2.4.1 Transmission

Transmission of CMV is not completely understood, but appears to require close contact with an individual who is excreting virus in urine, saliva, or other body fluids. Sexual contact is an important mode of transmission in some populations, such as homosexual men. Cytomegalovirus can also be transmitted by transfusion of cellular blood products or transplantation of infected organs.(Kinney et al. 1985) Infants can be infected in utero or in the perinatal period through ingestion of infected breast milk or by other exposures.

There has also been no evidence of an increased rate of CMV acquisition among health care workers who have frequent contact with HIV-infected patients, despite the high rate of CMV disease in these patients.(Gerberding et al. 1987; Kuhls et al. 1987)
2.4.2 Immunosuppressed Patients

In a review of four studies dealing with the development of CMV disease after kidney transplantation, Ho calculated that an average of 83% of individuals (range 78% to 88%) developed symptomatic disease after primary infections, and that an average of 44% (range 20% to 69%) developed symptomatic disease following reactivation of CMV infection. (Ho, 1991) Homosexual, HIV-infected men who excrete CMV in the semen have an increased rate of CMV disease, and this rate is increased further if more than one strain of CMV is shed in semen. (Leach et al. 1994)

Up to the early 1990s there was an increasing incidence and prevalence of CMV disease among patients with AIDS. (Hoover et al. 1993; Montaner et al. 1994; Gallant et al. 1992; Katz et al. 1994) In a study of 844 HIV-infected men, Hoover and associates found that CMV disease eventually developed in 44.9% of the individuals who had received prophylaxis against Pneumocystis carinii pneumonia. (Hoover et al. 1993) Since the mid 1990's the incidence of CMV retinitis has been falling in the developed world with the introduction of highly active antiretroviral therapies (HAART). (Palella et al. 1998) At the end of 1998, however, there has been a trend in a few US centres towards a slight increase in the number of new cases of CMV retinitis. It is not clear as yet whether this is a transient change or may reflect the increasing pool of HIV positive patients. Although, with the introduction of HAART the rate of patients with CMV retinitis is decreasing, the pool of HIV infected individuals itself is still growing, the net balance of which may result in an increase in the incidence of CMV disease again.
2.4.3 Ocular Disease

CMV retinitis will develop in 5% to 30% of newborns with clinically-apparent congenital CMV infection. Cytomegalovirus retinitis has not been seen in infants with perinatally acquired CMV infection, even among those who develop symptomatic disease. (Stagno et al. 1977) Maternal antibodies, if present, may offer some protection against disease in these cases.

2.4.4 Cytomegalovirus Retinitis in Patients with AIDS.

Cytomegalovirus retinitis is the most common ocular infection in patients with AIDS in the United States. (Holland et al. 1983; Palestine et al. 1991; Jabs et al. 1989; Schuman et al. 1987) The reported prevalence of AIDS-related CMV retinitis, in the pre-HAART era, varied from 4% of ambulatory patients (primarily intravenous drug abusers) (Rosenberg et al. 1983) to 34% of eyes in an autopsy series of male homosexuals. (Pepose et al. 1985) Cytomegalovirus retinitis is uncommon among African patients with AIDS, possibly due to death from other opportunistic infections before CMV disease can occur. (Kestelyn, 1990)

CMV retinitis is usually a late manifestation of HIV disease. Although CMV retinitis is an "index disease" for AIDS (meaning that, in the absence of other risk factors, its presence alone is sufficient for making a diagnosis of AIDS), (Centers for Disease Control, 1987) only about 2% of patients with AIDS have CMV retinitis as the first and only manifestation of the syndrome. (Sison et al. 1991)

With the introduction of HAART in the 1990s, there has been a fall in the incidence of CMV retinitis in the USA, and there is also a reducing proportion of Caucasian homosexual males of the total new presentations of CMV retinitis. In a
study by Palella and associates they found that the incidence of cytomegalovirus retinitis declined from about 17 per 100 person-years to less than 4 per 100 person-years by mid 1997. (Palella et al. 1998)

Cytomegalovirus retinitis is reported less commonly among HIV-infected children than among HIV-infected adults. (Dennehy et al. 1989) Cytomegalovirus retinitis in HIV-infected children generally does not develop for several years after birth, which reflects its association with declining immune function. Although CMV retinitis has been reported in HIV-infected infants, (Dennehy et al. 1989; Jonckheer et al. 1990; Levin et al. 1989; Salvador et al. 1993) its occurrence at birth is not diagnostic of AIDS, since it may be a manifestation of congenital cytomegalic inclusion disease.

Cytomegalovirus retinitis is more common among patients with AIDS than among other severely immunosuppressed individuals. Non-HIV infected groups that may develop CMV retinitis include recipients of heart (Quinlan and Salmon, 1993; Pollard et al. 1980), renal, (Pollard et al. 1980) or bone marrow transplants; (Coskuncan et al. 1994) and patients with a variety of malignancies, including Hodgkin disease, (Moeller et al. 1982) other lymphomas, (Pollard et al. 1980) leukemia, (Keith and La Nauze, 1980), and solid tumors. (Pollard et al. 1980) The occurrence of CMV retinitis in patients with malignancies is usually associated with the use of immunosuppressive chemotherapy. Cytomegalovirus retinitis has also been associated with the use of systemic corticosteroid and other immunosuppressive therapies. (Berger et al. 1979)
2.5 Risk Factors

Acquired CMV infection usually results from close contact with individuals who are shedding virus. Demographic factors associated with increased rates of virus transmission and congenital disease are presented in the Epidemiology section. Less commonly, infection results from transfusion of blood or transplantation of organs from infected donors.

The primary risk factor for the development of serious clinical CMV disease (other than cytomegalic inclusion disease in newborns) in newly infected or latently infected individuals is immunosuppression.

2.5.1 Ocular Disease

CMV retinitis occurs only in severely immunocompromised patients. There appears to be a relationship between CMV retinitis and CMV viraemia in patients with AIDS; Salmon and associates found that CMV viraemia at the development of AIDS was predictive of tissue-invasive CMV disease; 14 of 28 patients (50%) with positive CMV blood cultures developed disease, after a mean interval of 7.7 ± 6.3 months, in contrast to only four of 43 patients (9.3%) with negative blood cultures after a mean interval of 11.8 ± 3.8 months (p<0.001).(Salmon et al. 1990) Survival and mean CD4+ T-lymphocyte counts were not different between groups. Zurlo and associates also found that CMV viraemia and viruria in patients with AIDS was statistically associated with development of tissue-invasive CMV disease, but pointed out that the positive predictive values of CMV viraemia (35%) and CMV viruria (28%) for development of disease within 6 months are relatively low.(Zurlo et al. 1993) Fiala and associates also suggested a relationship between the duration of
CMV viraemia and development of CMV retinitis in organ transplant recipients. (Fiala et al. 1977)

Many workers have explored the relationship between systemic viral load and CMV disease by using the shell vial, antigenaemia assay or by PCR. Gerna and co-workers first showed that CMV-related symptoms in AIDS patients were generally found when the number of infected polymorphonuclear cells exceeded 50 per $2 \times 10^5$ cells, as determined by the shell vial assay or the antigenemia assay. (Gerna et al. 1990) By using quantitative PCR, two studies have shown that CMV DNA load in leukocytes of AIDS patients with CMV retinitis was significantly higher than the viral burden in cells of patients with asymptomatic CMV infection with no retinitis. (Rasmussen et al. 1997; Boivin et al. 1997) Similar results have been found in plasma samples for the detection of CMV DNA by PCR assay. (Shinkai et al. 1997) Recent studies have evaluated the use of CMV DNA load for the prediction of CMV disease in HIV-infected patients. Bowen and co-workers found a high risk (relative risk 20.15) of CMV disease in HIV-infected patients with CD4 counts of less than 50/μl who were PCR positive at baseline. (Bowen et al. 1997) In a study by Spector et al. PCR positivity at baseline was associated with CMV disease (3.1-fold increase for each log rise in viral load). (Spector et al. 1998) Other studies using either using PCR or antigenaemia assay have found similar results. (Rasmussen et al. 1997; Francisci et al. 1995)

2.5.1.1 CMV Retinitis in Patients with AIDS.

The only systemic risk factor, other than CMV DNA load, that has been clearly associated with development of CMV retinitis in HIV-infected patients is a
low CD4+ T-lymphocyte count. (Pertel et al. 1992; Kuppermann et al. 1993) The infection almost always occurs in patients with counts less than 50 per μl; the mean counts reported for patients with CMV retinitis have been 8 to 15.6 per μl. (Pertel et al. 1992; Kuppermann et al. 1993)

In a retrospective study of 135 patients, 26 of whom developed CMV retinitis during 27 months of follow-up, Pertel and associates found that the odds ratio for developing CMV retinitis was 4.62 (p=0.002) for patients with baseline CD4+ T-lymphocyte counts of 0-50 per μl when compared to those with counts of 101-250 per μl. (Pertel et al. 1992).

Despite its strong association with low CD4+ T-lymphocyte counts, CMV retinitis has been seen occasionally in patients with counts above 100 per μl. (Hochster et al. 1990; Crowe et al. 1991; Fekrat et al. 1995) An association has also been identified between CMV retinitis and low CD8+ T-lymphocyte counts, (Lowder et al. 1995) but it could not be determined from initial studies whether low CD8+ T-lymphocyte counts place a patient at risk independent of CD4+ T-lymphocyte.

As discussed in the Epidemiology section, CMV retinitis may be more common in homosexual patients with AIDS than in other risk groups. As discussed in the Pathogenesis section, the retinal microvasculopathy associated with HIV infection may be associated with the increased prevalence of CMV retinitis in patients with AIDS when compared to other immunosuppressed populations. Cotton-wool spots, the most common clinical manifestation of the microvasculopathy, are more common in patients with the full AIDS illness than in other HIV-infected individuals, and are
frequently seen in patients prior to the development of CMV retinitis, (Holland et al. 1983) and their presence has been found to be associated with an increased risk of developing CMV retinitis. (Jabs, 1995) Other risk factors under study include absence of specific CMV immunity, as measured by lymphoproliferative assays, and specific CMV strains.

The identification of risk factors for CMV retinitis in HIV-infected patients makes screening programs for the identification of asymptomatic disease more practical. In the pre-HAART era Kuppermann and associates advocate examining patients with CD4+ T-lymphocyte counts < 50 per μl every 3-4 months. (Kuppermann et al. 1993) In this prospective study, all 14 patients found to have previously undiagnosed CMV retinitis on screening examination reported no symptoms, although several patients actually had visual changes when instructed how to look for them. In contrast, MacGregor and associates found no asymptomatic CMV among 78 patients with CD4+ T-lymphocyte < 100 per μl. (MacGregor et al. 1995)

Although other investigators have advocated similar screening programs, there have been no data upon which to base the recommended intervals, nor has there been studies to determine whether long-term visual outcomes are affected by screening programs. Others have suggested patient education programs regarding early symptoms of disease as a more practical alternative to wide-spread screening. (Holland, 1992) Since it will be impossible to determine how long CMV retinitis remains asymptomatic, rational decisions regarding the value of screening or appropriate screening intervals will require a study of outcomes, and the evaluation in different subgroups, such as HAART failures.
2.6 Clinical Features

2.6.1 Immunosuppressed Patients

Cytomegalovirus is the most common life-threatening opportunistic viral pathogen in patients with AIDS, (Jacobson and Mills, 1988) and in one autopsy series, over half of patients dying from any AIDS-related complication had CMV infection of at least one organ. (Morinelli et al. 1992) As prophylaxis regimens against *P. carinii* pneumonia have become widespread, CMV infection has emerged as the most frequent initial AIDS-related illness. (Hoover et al. 1993) In the post-HAART era the incidence of CMV disease, as with other opportunistic infections, has been declining in the USA (Palella et al. 1998) and other developed countries.

It is estimated that up to 20% of patients with AIDS will develop CMV related-gastrointestinal disease. (Dieterich et al. 1993) Cytomegalovirus oesophagitis is a common cause of dysphagia in patients with AIDS and colitis/ileitis has been estimated to develop in 10% of patients with AIDS in the pre-HAART era. Cytomegalovirus is an infrequent cause of hepatitis and biliary tract disease. (Blanchard, 1992)

Cytomegalovirus can be detected in culture from a high proportion of lung tissue or bronchial fluids obtained from patients with AIDS; in one study, 43% of specimens were culture positive. (Broaddons et al. 1985) Cultures cannot distinguish between colonization by CMV and clinically significant infection, however. (Millar et al. 1990) Furthermore, the presence of inclusion bodies in the lung does not
necessarily implicate CMV as the cause of clinical disease, unless other causes have been excluded. (McKenzie et al. 1991) Only 4% of patients with AIDS and pneumonitis have been found to have CMV as the sole pathogen. (Murray et al. 1984) In contrast, CMV pneumonitis in immunosuppressed solid organ or bone marrow transplant recipients is common, and isolation of CMV from lung tissue or bronchial fluids is associated with a poor prognosis in bone marrow transplant recipients. (Meyers et al. 1982)

Cytomegalovirus infection of the nervous system in patients with AIDS may cause a number of disorders, including meningoencephalitis, polyradiculomyelitis, and peripheral neuropathy. (de Gans et al. 1990; Masdeu et al. 1988; Said et al. 1991) The role of CMV in the AIDS dementia complex is uncertain, but it is probable that HIV itself, rather than CMV, is the main causal agent. (Navia et al. 1986)

Other disorders attributed to CMV in patients with AIDS have included adrenalitis, (Glasgow et al. 1985) thyroiditis, (Frank et al. 1987) epididymitis, (Randazzo et al. 1986) and dermatitis, (Thiboutot et al. 1991)

2.6.2 Ocular Disease

Ocular CMV infection is primarily a disease of the retina, although there have been isolated reports of infection involving other ocular structures, as described at the end of this subsection. Detailed information about CMV retinitis is derived almost exclusively from patients with AIDS; possible differences in CMV retinitis between patients with AIDS and others at risk are identified in the discussion below.

Cytomegalovirus Retinitis.
The diagnosis of CMV retinitis is usually based on clinical findings. Untreated disease can have a variety of clinical presentations, with variable amounts of retinal whitening or opacification (due to oedema and necrosis), retinal haemorrhage, and vascular sheathing. Two distinct clinical types of CMV retinitis have been described, based on retinal characteristics. The "fulminant/oedematous" variant corresponds to the classic appearance of disease, which was recognized before the AIDS epidemic. These lesions tend to have the following characteristics: dense confluent areas of retinal opacification (through which choroidal details cannot be seen) involving both the border and central areas of the lesions; and the absence of clear central atrophic areas (unless the lesions are large, involving 25% or more of the retina). (see Figure 2.1)

Other suggestive, but not definitive, characteristics of fulminant/oedematous lesions are the following: location along vessels; haemorrhage sufficient to obscure underlying choroidal or retinal detail; and inflammatory vascular sheathing.

The "indolent/granular" variant is defined by the following characteristics: only faint, grainy opacification of the retina (through which choroidal details can be seen); only trace (punctate) or no haemorrhage; and no inflammatory vascular sheathing. (see Figure 2.2)

Other suggestive, but not definitive, characteristics of indolent/granular lesions are the following: circular or oval shape; location not overlying arterioles or venules; and opacification of the lesion border only, with the presence of a clear atrophic central area.
Both lesion variants have an irregular, dry-appearing, granular border, which is the most characteristic feature of CMV retinitis. There can be a variable number of distinct "satellite" lesions, which are considered to be encompassed within the lesion border. (see Figure 2.3)

Other clinical features that suggest a diagnosis of CMV retinitis include relatively slow enlargement of lesions and minimal vitreous humor and anterior chamber inflammatory reactions. These two variants seem to exist at the ends of a disease spectrum, and many lesions cannot be classified as one clinical type or the other; they are considered to be "indeterminant" in type.

In occasional patients, severe retinal periphlebitis has been the most prominent feature of AIDS-related CMV retinitis. (Geier et al. 1992; Spaide et al. 1992) Such patients have foci of CMV retinitis typical of the fulminant/oedematous variant somewhere in the fundus, but also have dense inflammatory sheathing of vessels throughout the retina, even remote from the site of infection. Perivascular inflammatory cells remote from foci of CMV-induced retinal necrosis can be found in many patients, (Holland et al. 1983) however, and those who appear to have frosted branch angiitis can be thought of as having an extreme form of the fulminant/oedematous variant of disease. In the post-HAART era patients may present with very indolent CMV retinitis lesions, with mild or no clinically evident border opacity that may gradually enlarge. With immune reconstitution, inflammatory complications may become present such as epiretinal membranes, cystoid macula oedema and vitritis. (Karavellas et al. 1998; Zegans et al. 1997)
Figure 2.1

Fulminant/oedematous variant of untreated CMV retinitis following the major retinal vessels in the right eye of a patient with AIDS.

Figure 2.2

Indolant/granular variant of CMV retinitis in the peripheral retina of a patient with AIDS.
Figure 2.2

CMV retinitis in the superior right eye. There are a number of satellite lesions at the border between infected and normal retina (arrow).

![Image of CMV retinitis](image)

Figure 2.4

Active CMV retinitis (left figure below) and inactive CMV retinitis following treatment (right figure below). The border of the lesion has no opacity. The central portion of the lesion is characterised by gliosis and mild pigment stippling.

![Images of active and inactive CMV retinitis](image)
2.7 Pathology

Cytomegalovirus infection occurs in a variety of tissues and cell types, but infection of macrophages and endothelial cells is seen frequently in vivo. A unique histopathologic finding in tissues infected by CMV is the presence of giant, or cytomegalic, cells, which classically have type A intranuclear inclusions.(Cowdry, 1934)

The cytomegalic cells are typically two to four times larger than surrounding cells, with a large round-oval nucleus that is often eccentrically displaced. The eosinophilic nuclear inclusion may be surrounded by a clear halo, giving rise to an "owl's eye" appearance. Basophilic granular inclusions may also be found in the cytoplasm. The origin of the cytomegalic cells is unresolved. Since cytomegalic cells are frequently associated with ductal epithelium they may be of epithelial origin, even though epithelial cells are not permissive to CMV in vitro.(Ho, 1991)

2.7.1 Ocular Disease

A number of publications have described the histopathologic features of CMV retinitis in patients with AIDS(Bachman et al. 1982; Pepose et al. 1985; Holland et al. 1983; Seregard, 1994; Palestine et al. 1984; Jensen et al. 1984; Jensen and Klinken, 1989) and other immunodeficiency states.(De Venecia et al. 1971) Electron microscopic and immunohistochemical studies identify CMV viral particles and antigen in a patchy distribution throughout all layers of the retina and occasionally in contiguous RPE cells.(Pepose et al. 1985) Infection results in retinal necrosis with
subsequent complete disruption of all retinal layers. Cytomegalic cells can be seen throughout the necrotic retina. There may be a sharp demarcation between normal and necrotic retina, or there may be a transition zone containing isolated cytomegalic cells and partially disrupted retinal elements.

It is uncommon to find CMV in intraocular tissues other than the retina. Cytomegalovirus has been detected rarely in the iris stroma and ciliary body of patients with AIDS. (Pepose et al. 1985; Teich et al. 1988; Daicker, 1988) Viral antigens are rarely identified in choroidal tissue of patients with AIDS and CMV retinitis. (Pepose et al. 1985; Rodrigues et al. 1983) When present in the choroid, viral antigen has been found in association with vessels and is not always adjacent to areas of CMV retinitis, suggesting that the virus reaches the choroid through independent hematogenous spread, rather than by extension of infection from retina. (Pepose et al. 1985)

Cells with CMV viral inclusions may be seen in the choroidal and retinal vascular lumina. (Christensen et al. 1957; Tsukahara et al. 1966) In immunosuppressed patients without HIV infection, the spread of disease along vessels has been attributed to infection of vascular endothelial cells, which break free and travel through the retinal circulation. (De Venecia et al. 1971) In support of this hypothesis, inclusion bodies have been identified in retinal endothelial cells of organ transplant patients with CMV retinitis. In initial studies on patients with AIDS and CMV retinitis, endothelial cell inclusions were reported rarely in choroidal tissue, (Rodrigues et al. 1983; Bachman et al. 1982) but CMV antigen was not identified in retinal endothelial cells by immunohistochemical techniques even in vessels that travel through areas of retinitis. (Holland et al. 1983), and so it was
hypothesised that virus spread adjacent to vessels in the anatomic spaces of Virchow. (Holland and Shuler, 1992) However, studies by Rao and associates (Rao et al. 1998) in the late 1990’s studying autopsy eyes of AIDS patients with and without clinically apparent CMV retinitis, with anti-CMV antibodies to immediate early, early and late antigens to determine infected retinal cells, found immunoreactive cells present predominately in Muller and perivascular glial cells. This study found that within the CMV lesions most of the retinal capillaries were devoid of endothelial cell, but some vessels at the advancing margin of retinal necrosis showed the presence of viral protein in the endothelial cell. (Rao et al. 1998) Productive infection was also noted in primary cell culture of endothelial cells. This study suggests that retinal vascular endothelial cells are the primary target for CMV infection with subsequent spread to glial and Muller cells and then to other retinal elements including retinal pigment epithelium.

In most cases, CMV infection elicits only a mild cellular inflammatory response in the retina. (Tsukahara et al. 1966; Hennis et al. 1989; Newman et al. 1983; Bachman et al. 1982; D'Amico et al. 1988; Grossniklaus et al. 1987; Jensen et al. 1984) It is usually characterised by a sparse infiltrate of mononuclear cells in both patients with AIDS and those who are immunosuppressed by other mechanisms. However, in the post-HAART era, a more marked clinical inflammatory response, in terms of vitritis and epiretinal membranes is more commonly noted. (Karavellas et al. 1998; Zegans et al. 1997) In addition, however, 22% to 50% of patients with AIDS and CMV retinitis are found to have foci of neutrophilic infiltrates in retinal tissue at autopsy. (Pepose et al. 1985; Palestine et al. 1984) This finding is not typical of CMV retinitis in patients without HIV infection. The difference has been attributed to
intact granulocyte function and chemotaxis in patients with AIDS, in contrast to
infants, organ transplant recipients, and patients with malignancies who can have
more severe quantitative or qualitative granulocyte dysfunction. (Pepose et al. 1985)

A variety of stimuli (CMV antigen, immune complex deposition, and tissue
necrosis) may be responsible for the production of chemotactic factors leading to
inflammatory cell infiltration. Immunochemical studies have revealed deposition of
IgG, IgA, and, to a lesser extent, IgM and C3c in retinal tissue and within retinal
arteriolar walls. (Pepose et al. 1985) Many IgA-bearing plasma cells were present in
one case. (Holland et al. 1983) There is poor correlation between the distribution of
tissue-bound immunoglobulins, acute inflammatory cells, and CMV antigens. (Pepose
et al. 1985) It is therefore possible that the acute inflammatory reaction present in
some eyes may be in response to stimuli other than CMV.

The perivascular inflammatory sheathing that is seen clinically in cases of
CMV retinitis is composed of neutrophils, and is consistent with an immune complex-
mediated vasculitis, although recent evidence suggest that human CMV infected
endothelial cells are themselves a potent C-X-C chemokine. (Grundy et al. 1998)
Possible reasons for CMV infected cells to attract neutrophils are to control virus
dissemination, or to modulate host immune response. CMV antigen was not
identified in the affected vessels in initial studies (Pepose et al. 1985; Holland et al.
1983) possibly due to loss of endothelium in affected lesions, however CMV proteins
have been found in retinal endothelium in vessels located at the advancing margin of a
lesion. (Rao et al. 1998)
A secondary choroiditis consisting of mononuclear cells or neutrophils may occur subjacent to areas of CMV retinitis without identifiable viral antigens in the inflamed choroid. (Pepose et al. 1985) In areas where CMV antigens can be identified in choroid, only a minimal cellular inflammatory reaction is elicited. (Pepose et al. 1985)

In the acute stage of disease, there may be subretinal fluid with exudative retinal detachment. (Pepose et al. 1985) In the later stages of CMV retinitis, the necrotic retina thins with formation of multiple retinal holes, which predispose patients to retinal detachment. Eventually the necrotic retina is replaced by a thin gliotic membrane. At this stage, CMV particles cannot be identified in the eye histologically.

2.8 Pathogenesis

Cytomegalovirus (CMV) disease can result from primary exogenous acquisition of the virus, from congenital infection, or from reactivation of endogenous/latent virus, but the pathogenesis of CMV infection in each setting is yet to be fully elucidated.

2.8.1 Spread of the Virus

Following primary infection, CMV is disseminated by the blood stream to various organs, with virtually all virus being cell-associated during this process. Cytomegalovirus can be recovered from peripheral blood granulocytes, which may
contain large amounts of viral DNA. (Cox et al. 1975; Fiala et al. 1977; Martin et al. 1984; Saltzman et al. 1988) Molecular studies have also found the viral genome in monocytes, although they are usually culture negative. In some studies, viral RNA is identified only in monocytes, (Turtinen et al. 1987) while in other studies viral RNA is found in both monocytes and neutrophils. (Dankner et al. 1990; Gerna et al. 1992) Late viral antigens can usually be detected in the nuclei of neutrophils during viraemia, which has been termed "CMV antigenemia". (Grefte et al. 1993)

Once clinically apparent CMV disease develops, viraemia is virtually always present. Patients with AIDS and visceral organ CMV disease, especially colitis, have 20 to 25 fold greater amounts of CMV DNA in their granulocytes than those with only CMV retinitis or CMV viraemia without organ involvement, and the amounts of viral DNA in granulocytes of AIDS and organ transplant patients with CMV retinitis were low (median 22 pg) and of similar magnitude. (Saltzman et al. 1992) Presumably, this high-grade viraemia reflects the greater amount of virus replication and tissue injury that occurs with visceral organ infection when compared to retinal infection. Similar patterns were seen in solid organ transplant recipients with visceral organ CMV disease when compared to those with viraemia but no organ involvement. In contrast, the amounts of CMV DNA in granulocytes of bone marrow transplant recipients were low whether or not visceral organ CMV disease was present, possibly reflecting a modulating effect of graft-versus-host disease. Cytomegalovirus DNA, but not infectious virus, has also been detected free in the plasma during viraemia. (Brytting et al. 1992; Wolf and Spector, 1993)

Following initial infection, CMV is disseminated to many organs. It is commonly found in the urine, secretions from the oropharynx and vagina, and in
In humans, CMV has been detected in a variety of organs including the kidney, spleen, salivary gland, brain, inner ear, lungs, and gastrointestinal tract. Using immunohistochemical techniques, CMV has been detected in endothelial, epithelial, smooth muscle, and parenchymal cells.

Organs contain infectious virus for variable lengths of time, depending on the host immune status. If the host is immunocompetent, the infection is usually subclinical, despite diffuse organ involvement. In congenitally infected newborns and immunosuppressed older children and adults, replicating CMV is found in many organs, and there is a prolonged period of virus excretion in urine and saliva.

In the immunosuppressed host, retinal infection can occur at the time of primary infection or after reactivation of latent CMV. Whether latent CMV exists in retinal cells remains to be clarified. It is assumed, however, that in patients with chronic infection, virus reaches the eye to cause CMV retinitis by hematogenous spread after reactivation elsewhere in the body. Sludging of blood flow and damage to the retinal microvasculature, which occur commonly in HIV-infected patients, may increase the contact time between CMV-infected leukocytes, which might become trapped in abnormal capillary nets; this hypothesis is discussed in the subsection "Factors Affecting Disease Severity" below.
2.8.2 Chronic Infection and Latency

CMV has an affinity for the salivary glands, where it produces a persistent infection with shedding of virus into the saliva, which may be a means of virus transmission in a population. (Ho, 1991) Such excretion may occur in normal individuals without CMV disease. (Griffiths and Grundy, 1988) Cytomegalovirus may also persist in the body after primary infection in a latent state in which the viral genome is present but gene expression is either limited or does not occur at all, and infectious virus particles are not produced. (Stevens, 1989)

From evidence in human and animal studies, it is probable that all seropositive individuals harbor latent virus that can reactivate. (Ho, 1991) Knowledge about mechanisms and sites of latency is incomplete, but cellular blood elements are one probable site. Information regarding the location of latent CMV is indirect and is based primarily on epidemiologic data, in which CMV is transmitted by blood transfusion and organ transplantation. Seroconversion that occurs after blood transfusion is proportional to the amount of blood transfused; (Prince et al. 1971) although the rates are less than 1% per unit of blood. (Kane et al. 1975; Tolpin et al. 1985; Griffiths and Grundy, 1988) The rate of seroconversion decreases with the transfusion of leukocyte-poor blood. (Lang et al. 1977) The identification of CMV DNA in blood leukocytes (especially mononuclear cells) by PCR techniques also suggest that they are sites of latency, although these results are difficult to interpret because of positive results in some seronegative controls. (Cassol et al. 1989; Stanier et al. 1989) Viral RNA can be detected in T-lymphocytes by in situ hybridization techniques during latency, in the absence of viral antigens or infectious particles. (Cassol et al. 1989; Stanier et al. 1989)
Cytomegalovirus may also persist in a variety of other cell types in various organs. Human CMV DNA has been demonstrated in monocytes, endothelium, macrophages, and smooth muscle in arteries of individuals with and without atherosclerotic disease. (Petrie et al. 1987; Yamashiroya et al. 1988; Hendrix et al. 1990) It is probable that there is more than one cell type in which CMV latency can occur.

The host's failure to eradicate CMV may be attributable to either ineffective immune response or escape mechanisms on the part of the virus, such as downregulation of cell surface viral molecules. (Bruggeman, 1993) A discrepancy in the sites of local virus production and latency has been observed in a murine model, (Balthesen et al. 1994) which probably reflects the cytocidal nature of active CMV infection, which thereby prevents latency in those tissues in which productive infection occurs.

2.8.3. Immune Response

Host defense to CMV may involve innate non-specific immune mechanisms, as well as specific humoral and cellular immunity. (Sissons, 1986) Since CMV has the ability to persist and remain latent, it is clear that the immune defenses are only partly successful in combating CMV infection. In some cases the immune response may even contribute to pathology.

Mononuclear phagocytes and natural killer (NK) cells appear to be important in early resistance to infection. If mice are depleted of NK cells, there is an increased severity and duration of murine CMV infection. (Shanley, 1990) It is not clear whether the same phenomenon is true for man, but in rare patients with a depletion in
NK cells, there is an increase in herpetic infections, including CMV infection. (Biron et al. 1989)

Immunohistochemical studies on CMV-infected retina in patients with AIDS have revealed the expression of an inducible form of nitric oxide synthetase in glial cells. (Dighiero et al. 1994) Host production of toxic nitrogen intermediates is a known antimicrobial mechanism.

2.8.3.1 Humoral Immunity.

Humoral immunity is probably not the main defense against CMV, since CMV disease occurs most commonly in patients who have depressed cell-mediated immunity, and develops despite the presence of circulating antibodies. Nevertheless, there is evidence that humoral immunity does play some protective role; the presence of maternal antibody appears to protect newborns from the development of symptomatic CMV disease. (Stagno et al. 1982) and immune globulin may be protective against the development of pneumonitis in bone marrow transplant recipients. (Winston et al. 1990)

IgA, which is produced in 90% of primary CMV infections, may protect epithelial surfaces. (Levy and Sarov, 1980) In immunocompetent patients, both IgG and IgM are produced early in the primary infection; IgG antibodies persist throughout life, while IgM antibodies disappear after the first month of a primary infection, although they may reappear during reactivation. Antibodies to a number of specific CMV proteins can be identified, (Landini et al. 1988) but it has been difficult to identify the importance of reactions against specific proteins because of the complex nature of the polypeptide profile of CMV. Antibody to phosphoprotein
p150, a component of the viral matrix, is a useful marker of infection, (Landini et al. 1986) but may not be detectable early. (Ripalti et al. 1989) Other immunogenic proteins are the 28, 35, and 52-KDa DNA-binding proteins. The envelope glycoproteins gC11 complex, gB, and gH are also known to be immunogenic, (Rasmussen et al. 1991) although it is not clear whether they produce protective immunity. Antibody to gB may have some protective effect; it develops concurrently with the initial IgG and IgM response, and its levels increase during secondary infections. (Marshall et al. 1994) Antibody to gH is detected in HIV-seronegative individuals transiently after recent infection; in contrast, HIV-infected individuals may have high sustained levels of antibodies to gH, possibly in response to multiple episodes of CMV reactivation or reinfection. (Rasmussen et al. 1991; Rasmussen et al. 1994)

2.8.3.2 Cell-Mediated Immunity and Cytokines.

T-lymphocytes play the most important role in host defences against CMV. The mechanisms by which T-lymphocytes mediate defence against CMV are multiple, but include cytolysis of infected cells and cytokine production. Cell-mediated immunity to CMV has been studied by tests looking at the effector arm of the immune response, by tests looking at antigen recognition, and by tests that look at both functions. Tests of lymphocyte blastogenesis have been used most often to study recognition of CMV antigen. Most healthy seropositive individuals have a positive response, (Pollard et al. 1978) indicating the presence of circulating T-lymphocyte memory cells specific for CMV. In contrast, few congenitally or perinatally infected individuals have a positive response. (Pass et al. 1981) This failure to respond
typically recovers by age 3 to 5 years, when viruria stops. Impaired test response in
mothers has been shown to be a risk factor for intrauterine transmission of
CMV.(Stern et al. 1986)

Cytotoxic tests for CMV have been used to examine both recognition and
effecter functions. Cell-mediated cytotoxicity by CD8+ T-lymphocytes has been
strongly implicated in the control of CMV infection.(Quinnan et al. 1982) In studies
exploring the reactivation of latent MCMV on B-cell deficient mice Polic and co­
workers found a hierarchy of immune control functions of CD8+, NK, and CD4+
cells with reactivations being rare if only one of the lymphocyte subsets was removed,
but was evident after removal of a further subset.(Polic et al. 1998)

Cytomegalovirus infection is known to alter production of several different
cytokines. Cytomegalovirus infection of fibroblasts result in the release of IFN-β,
which induces upregulation of HLA Class I molecule expression, presumably on
nearby uninfected cells.(Grundy et al. 1988) This action may facilitate recognition of
viral antigens by the immune system. Conversely, other studies on CMV-infected
fibroblasts have shown a fall in HLA Class I molecule levels in the infected cells
themselves.(Grundy and Downes, 1993) Increased production of IFN-γ and tumour
ecrosis factor (TNF)-α has been shown to occurs in human CMV-infected fibroblasts
that are co-cultured with T-lymphocytes that have been activated in vivo.(Duncombe
et al. 1990) Interferon-γ has been shown to suppress murine CMV viral expression in
microglial cells of mice.(Schut et al. 1994) Elevation of serum interleukin (IL)-6, IL­
8, and C-reactive protein occur in patients with CMV infection.(Schwaighofer et al.
1994; Murayama et al. 1994) In immunosuppressed rats, TNF-α production has been
shown to be induced by replicating rat CMV in a variety of cell types. (Haagmans et al. 1994) Genes for immediate-early proteins of human CMV have been found to upregulate TNF-α expression, which may account for some of the inflammatory manifestations of CMV infection. (Geist et al. 1994) Vitreous humor from patients with AIDS and CMV retinitis contains increased levels of IFN-γ when compared to eyes from controls without HIV-infection who were undergoing vitreoretinal surgical procedures for a variety of reasons. (Mondino et al. 1990) Infection of fibroblasts and vascular endothelium by CMV increases the expression of adhesion molecules LFA-3 and ICAM-1 (Grundy and Downes, 1993) and HLA-DR on the cell surfaces. (Waldman et al. 1993; Scholz et al. 1992) The enhanced endothelial HLA-DR expression is probably mediated by IFN-γ release. (Waldman et al. 1993)

Cytomegalovirus induces transforming growth factor (TGF)-β1, which downregulates host immune responses and independently can enhance viral replication. (Michelson et al. 1994)

Human CMV UL146 strain infected cells have found to be a potent C-X-C chemokine, attracting neutrophils. It is unclear what benefit CMV infected cells have in attracting neutrophils, and it has been suggested that this may allow for the control of virus dissemination or as a decoy for the immune response. (Grundy et al. 1998) CMV-infected endothelial cells can recruit neutrophils by the secretion of C-X-C chemokines and can transmit the virus to them by direct cell-to-cell contact and during neutrophil transendothelial migration, suggesting that the neutrophil-endothelial cell interaction plays an important role in virus dissemination in vivo.
2.8.4 Effect of Cytomegalovirus on Host Immunity.

Human cytomegalovirus infection has evolved many mechanisms to evade and interfere with the host’s immune responses, including reduction in cell surface expression of major histocompatibility complex (MHC) class I, coating itself with beta 2 microglobulin,(McKeating et al. 1987) and prevention antigen presentation to CD8+ cytotoxic T-lymphocytes.(Barnes and Grundy, 1992; Beersma et al. 1993) Human CMV encodes 4 proteins that interfere with class I HLA transport and presentation. As a consequence of this class I HLA downregulation, the cell should become susceptible to NK cell lysis.(Ljunggren and Kserre, 1990) A human CMV gene product, UL18, is able to act as a broad spectrum NK cell decoy by binding to NK-inhibitory receptor that normally recognises MHC class I and protects the uninfected host cell from NK attacks.(Hassan-Walker et al. 1998) Cytomegalovirus infection is known to have an immunosuppressive effect on the host. Although the exact basis for this effect has yet to be established, the presence of CMV in circulating cells suggest that virus-leukocyte interactions play a key role in this immunosuppression.(Turtinen et al. 1987)

2.8.5 Factors Affecting Ocular Disease Severity.

A variety of factors may be responsible for the difference in frequency of CMV retinitis between HIV-infected patients and those who are immunosuppressed for other reasons; they include the possibility of more severe differences in the nature of immunosuppression among HIV-infected individuals, more severe or prolonged viraemia, or transactivation of CMV by HIV in retinal tissue, as discussed in the following subsection.
Also, the frequent development of CMV retinitis in patients with AIDS may be related in part to the retinal microvasculopathy that is associated with HIV infection. (Pepose et al. 1985; Newsome et al. 1984; Freeman et al. 1989; Glasgow and Weisberger, 1994). Ultrastructural studies of retinal capillaries have demonstrated microaneurysms, narrowing and occlusion of vascular lumina, basal lamina thickening, and swelling of endothelial cells and loss of pericytes with an increased ratio of endothelial cells to pericytes. This microvascular disease may facilitate access of virus to retinal tissue through damaged vessel walls. In an autopsy study, Glasgow and Weisberger have shown that these vascular changes are more severe in eyes with CMV retinitis (Glasgow and Weisberger, 1994); they hypothesised that the vascular changes may predispose to development of infection, but they could not rule out the possibility that the more severe vasculopathy is secondary to the CMV retinitis or that it may be an independent reflection of the more advanced state of AIDS in which CMV retinitis develops.

Hematogenous spread of virus to the eye is supported by the finding of CMV-like viral particles within macrophages in the choroidal circulation of an AIDS patient with CMV viraemia. (Palestine et al. 1984) Autopsy studies have shown aggregates of lymphocytes in vessels leading to areas of CMV retinitis. (Glasgow and Weisberger, 1994) Sludging of blood flow through the retinal circulation may also contribute to infection by increasing the contact time between circulating infected cells and retina. (Engstrom et al. 1990)
2.9 Treatment

Early attempts to treat CMV disease met with only limited success; agents included vidarabine, transfer factor, and IFN-α. (Ch'ien et al. 1974; Chou, 1986; Rytel et al. 1975; Pollard et al. 1980) The AIDS epidemic stimulated the development of several new antiviral drugs with anti-CMV activity. The first to become available were ganciclovir and foscarnet for intravenous and intravitreous administration; followed by oral ganciclovir, ganciclovir implants, cidofovir, and fomivirsin. In addition the non-CMV specific HAART regimes have been highly effective at controlling and preventing CMV retinitis. In 1999, the following agents were under clinical investigation for possible use in treatment of CMV retinitis: lobucavir, a cyclobutyl derivative of guanine that also acts by inhibition of DNA polymerase (Norbeck et al. 1990), and proganciclovir.

2.9.1 Monitoring Therapy

It was difficult to interpret and compare early reports regarding the effects of ganciclovir on CMV retinitis because investigators described the disease and its response to therapy with poorly defined terms, and they used a variety of unrelated outcome measures. In response to this problem, standardised nomenclature was adopted, and an algorithm for assessment of response to antiviral treatment was developed in anticipation of future clinical trials.1 (Holland et al. 1989)

---

1 Progression of disease is defined as the development of new lesions or the enlargement of preexisting lesions. Enlargement of preexisting lesions is most easily identified by the advancement of any segment of a lesion border toward previously uninfected retina. In clinical studies, an arbitrary
It is important to document the status of disease at diagnosis, and follow patients carefully for signs of change in their disease. At baseline examination, the following parameters are documented: best corrected visual acuity in each eye; the number of discrete lesions in each eye; the location of lesions; and the extent of disease. The location of lesions is identified using a system of zones. The location of lesions has important prognostic implications.²

The goal of treatment for CMV retinitis is to prevent CMV from destroying additional normal retinal tissue. This concept is the basis for the algorithm used to follow the course of disease and its response to treatment. (Holland et al. 1989) It utilises three parameters: development of new retinal lesions, enlargement of preexisting lesions, and change in opacification (whiteness) of lesion borders.

² Zone 1 lesions, or those in the macular and peripapillary areas, are considered immediately vision-threatening. Zone 3 lesions, because they underlie the vitreous base (a broad area of attachment between the retina and vitreous body), can be associated with vitreoretinal traction, and are more likely to result in retinal detachments.
The opacity of lesion borders is also monitored because it is believed to reflect viral activity. Change in the opacity of lesions is the most obvious effect of treatment, but is not a reliable surrogate for change in the size of lesions; (Holland et al. 1989) even minimally opaque lesions can continue to enlarge, with destruction of additional retina. Nevertheless for lesions that have not enlarged, change in border opacity from the previous examination, may help to predict eventual outcomes. (Holland et al. 1989) Increasing border opacity usually predicts eventual enlargement of lesions. Lesions that are enlarging on therapy will generally have a mildly opaque border, which has been called "smoldering retinitis." Comparison of serial retinal photographs is the best means of identifying the presence of subtle retinal opacities and changing opacification over time. If opaque lesion borders have not advanced, changes in therapy are not warranted; in some cases stable opacity of the border may represent gliosis with calcification or necrotic debris that has not cleared instead of active viral disease. (Keefe et al. 1992)

Other outcome measures, such as retinal detachments or visual acuity, are not considered to be direct measures of treatment efficacy. For example, active lesions in the anterior retina can continue to enlarge with little immediate effect on central vision. Conversely, a small lesion near the fovea can have a profound effect on vision, and even if treatment prevents further enlargement of a parafoveal lesion, vision may deteriorate further because of scarring and retinal traction, retinal detachment, or other changes that occur in the healing process. Preservation of vision is a measure of long-term treatment success, however.
2.9.2 Intravenous Therapy

Ganciclovir, foscarnet, and cidofovir suppress CMV replication, but do not eliminate virus from the eye. Electron microscopic studies of eyes with CMV retinitis that have been treated with ganciclovir show viral particles at the borders of lesions; the appearance of the particles is consistent with ineffective viral replication. (Pepose et al. 1987)

Based on early clinical experience with ganciclovir, a similar treatment regimen has evolved for all the intravenous drugs: (Gross et al. 1990; Henderly et al. 1987; Holland et al. 1987; Jabs et al. 1987; Palestine et al. 1986; Orellana et al. 1987; Jacobson et al. 1989; Collaborative DHPG Treatment Study Group., 1986) patients are given an initial "induction" course of therapy, to inhibit viral replication and prevent further enlargement of lesions, followed by life-long secondary prophylaxis or "maintenance" therapy to prevent disease reactivation.

2.9.3 Intraocular Therapy

Foscarnet, ganciclovir and fomiversin may be delivered to the eye by intravitreal injection. Intraocular devices may also be used for local drug delivery. Intraocular devices consist of a ganciclovir pellet encapsulated in plastics that releases drug at a constant rate for typically between 6 to 8 months. The median time to lesion border progression is longer (226 days) with intraocular devices than with any other currently available specific CMV treatment. The intraocular devices are sutured in through a pars plana incision and can be replaced when depleted of drug either at the same or at an alternate site. Although local therapies have certain advantages such as reduced cost, lack of systemic toxicity and need for indwelling catheters, there are
potential disadvantages, including increased risk of early retinal detachment, endophthalmitis, and the development of CMV disease in the fellow, uninvolved eye and in other organs. In addition intraocular implants may cause astigmatism and vitreous humor inflammation and haemorrhage that is usually transient. Local therapy is an important therapy option in selected patients such as those unable to receive systemic drug, or those with disease that is poorly controlled on maximal medical therapy. In patients with AIDS and CMV retinitis, oral ganciclovir in conjunction with a ganciclovir implant reduces the incidence of new CMV disease and delays progression of the retinitis, even with patients on protease inhibitor regimes. (Martin et al. 1999)

With the advent of highly active antiretroviral regimens, some patients who respond well (i.e. CD4+ T-lymphocyte count rises to more than 200) may be allowed a trial of discontinuation of their specific anti-CMV therapy, however the period of follow up of such patients has been short.

New therapeutic and monitoring strategies need to be developed with the introduction of local and HAART therapies. (Whitley et al. 1998) There is a need for a marker to differentiate between patients at risk of developing systemic CMV disease and who do not, in patients on local therapy alone. In patients on HAART therapy a way of determining which patients are still at risk of developing or reactivating their CMV retinitis is also needed.
Chapter 3 Cytomegalovirus DNA Load Measurements

3.1 Introduction

CMV retinitis is only one manifestation of disseminated CMV disease; autopsy studies have shown that all patients with CMV retinitis will have tissue destructive CMV disease in other organs as well. (Pepose et al. 1985) It is therefore possible that measures of CMV activity elsewhere in the body will have implications for ocular disease. Urine and blood cultures are of low positive- and negative-predictive value for the development of CMV disease in HIV-infected patients. (Salmon et al. 1990; Zurlo et al. 1993; MacGregor et al. 1995) In contrast, several other tests, such as quantitative antigenemia assays, branch chain DNA assays, and measurement techniques based on the polymerase chain reaction (PCR), appear promising in their predictive value for the development of CMV disease in HIV-infected patients. (Hansen et al. 1994; Francisci et al. 1995; Bowen et al. 1997; Dodt et al. 1997; Spector et al. 1998)

Previous studies have explored the relationship between CMV load and symptomatic CMV disease in transplant patients. There is substantial evidence that weekly measurement of the systemic CMV load during the first 3 months after solid organ transplantation is useful to predict CMV disease, and moderate evidence that measurement is useful in bone marrow transplantation. (Cassol et al. 1989, Wolf et al. 1993; Imbert-Marcille et al. 1995) It is possible that this relationship of CMV load and CMV disease exists in HIV-infected patients.
The purpose of these studies assessing quantitative virus load, is to see if rising or raised levels of CMV DNA in the peripheral blood precedes the development of new CMV disease or reactivation of CMV retinitis in HIV-infected individuals. We chose to base our study on the hybrid capture assay, because of potential low cost, ease of use, easy sample storage and preparation and reliability. Before undertaking the main study (see section 3.4) we evaluated the assay by comparing it to some other established methods of detecting CMV DNA in the peripheral blood (see section 3.3).

### 3.2 Quantitative assays for cytomegalovirus

Cytomegalovirus quantitation can be performed on blood and other body fluids. In blood samples CMV DNA can be recovered from both cellular elements (Boivin et al. 1997; Saltzman et al. 1988) (polymorphonuclear cells or monocytes) by a variety of methods including culture and in cell-free plasma or serum by PCR. (Hansen et al. 1994; Spector et al. 1992) Most studies show that the quantity of viral DNA in leukocytes is generally greater than in plasma for both transplant recipients and HIV infected patients. (Zipeto et al. 1995; Rasmussen et al. 1997; Wentworth and French, 1970; Gerna et al. 1994)

An overview of the main methods of quantitative measures of CMV in the blood are described below

#### 3.2.1 Quantitative viral cultures

The two main methods of quantitating CMV in culture are the plaque and shell vial assays. In the plaque assay serial dilutions of the specimen are inoculated onto fibroblast monolayers and after infection the cells are overlaid with a semisolid
medium. The virus spreads from cell to cell resulting in a plaque. The number of plaques can be counted under a microscope. The shell vial centrifugation culture assay system is a more rapid test. CMV is quantified either by keeping the viral inoculum constant and counting the number of infectious foci per shell vial or by inoculating serial dilutions of the sample and determining its titre.

The shell vial assay has been correlated with other, subsequent, CMV assays. Although the shell vial based assay correlates well with the pp65 antigenaemia assay in plasma and PMN, and PCR-based assays, the sensitivity of culture based assay is significantly lower than the other two methods. (Gerna et al. 1994)

The practical use of these assays is limited by the low sensitivity, and the rapid loss of viability of stored specimens compared to other methods. Other problems that may occur include poor ability of some CMV strains to form plaques, rapid loss of viability, lack of staining of monoclonal antibodies and nonspecific monolayer toxicity.

3.2.2 Quantitative pp65 antigenaemia assay

The pp65 antigenaemia assay consists of direct staining of polymorphonuclear cells (PMN) with monoclonal antibodies directed against the lower matrix protein pp65 (UL83). The results are expressed as the number of antigen positive cells relative to the number of cells used to make the slide or to quantify the number of positive cells per 50,000 cells. Studies have shown the antigenaemia assay to be more sensitive than culture methods and either as sensitive or less sensitive than PCR assay, depending on the various methods used for each assay. Because of the relatively low
frequency of p65 antigen-positive cells in most patients, a substantial degree of intra-assay variability can occur.

Advantages of the antigenaemia assay are its sensitivity, short processing time, and lack of requirement of a highly specialised laboratory. The disadvantages are the requirement for quick processing of the sample, the subjective component of slide interpretation and the relative time-consuming nature of the assay. As there are numerous modifications to the assay, some of which alter its sensitivity, standardisation of the assay would greatly improve the ability to compare study results.

3.2.3 Quantitative polymerase chain reaction assays

The relationship $Y=X(1+E)^n$, where $Y$ is the amount of PCR-amplified DNA, $X$ is the amount of target DNA prior to PCR, $E$ is the average efficiency of each cycle and $n$ is the number of amplification cycles, can be used to determine the relative or absolute amounts of target DNA or cDNA subjected to amplification. The concentration of an unknown quantity of target DNA in a clinical sample can be determined from a standard curve, generated by plotting logarithmic values of the PCR products against the logarithmic values of known quantities of target DNA (standards). The original number of viral copies present in a clinical sample can be determined by interpolating the values of amplified product into the standard curve. This strategy is valid if PCR is performed at the exponential part of the curve. However in most PCR procedures, the overall efficiency is less than 100% and the increase in amplicons only stays exponential for a limited number of cycles, after which the amplification reaches a plateau.
There are many different quantitative-PCR assay protocols that vary in many aspects including specimen types, primers and targets, quantitation standards and controls, reaction and amplification protocols, prevention and contamination, and signal generation systems.

Boivin and co-workers reported reliable results over a large dynamic range (25-25,000 copies of CMV DNA in a background of $10^5$ leukocyte) with intra- and interassay variabilities of 15 and 24% respectively.

Most QC-PCR protocols described so far have not been of immediate use in diagnostic virology labs because of their expensive and laborious detection procedures as well as their limited potential for batch testing.

3.2.4 Branched -DNA signal amplification assay

The bDNA signal amplification assay (Chiron Corp. Emeryville, CA) relies on signal amplification with bDNA multimers to quantify CMV directly from clinical specimens. The bDNA molecule provides multiple binding sites for an enzyme-labelled probe. Polymorphonuclear cells are isolated and incubated with a proteinase in lysis buffer, and the target probes are added. After incubation for 16 to 18 hours in a micro-titre plate, the bDNA is added: then the enzyme labelled probe and the chemiluminescent substrate are added. The complex is detected by a chemiluminescent substrate in which the light output is directly proportional to the sample input and the amount of DNA in the original sample. The bDNA assay can be used with blood, cerebrospinal fluid, semen, and frozen samples (provided PMN are separated within 8 hours of collection).
The lack of amplification makes this assay less prone to contamination. Disadvantages of this assay are the requirement for relatively large numbers of leukocytes and its relatively long processing time.

3.2.5 Solution Hybridization CMV DNA assay

The hybrid capture system (Digene Corp, Beltsville) is a solution hybridization assay that is based on signal amplification chemiluminescent detection. A specific CMV RNA probe complementary to about 17% of the genome is used to hybridise with the target DNA. (see Figure 3.1)

The resultant RNA-DNA hybrids are captured onto the surface of a tube coated with antibodies specific to RNA-DNA hybrids. The hybrids are immobilised in a tube and reacted with an alkaline phosphatase conjugated antibodies specific for the hybrids and detected with a chemiluminescent substrate. The signal is amplified as each RNA-DNA hybrid brings about 1000 antibody conjugate molecules, each of which is bound to three alkaline phosphatase molecules. The resulting signal is therefore amplified at least 3000-fold. The amount of light emitted is proportional to the amount of target DNA in the specimen.

The intra- and interassay coefficient of variation of the hybrid capture system have been estimated at 17.8% and 16.3% respectively. (Mazzulli et al. 1996)

More details about the hybrid capture assay are in the methods and discussion section of this chapter.

A summary of studies of quantitative assays for CMV load in the blood is shown in Table 3.1.
The Hybrid Capture CMV DNA Assay Probe contains 11,000 bp from Region 1 and 26,900 bp from Region 2.
**TABLE 3.1 Summary of studies of quantitative assays for CMV load in the blood**

<table>
<thead>
<tr>
<th>References</th>
<th>Number of patients</th>
<th>Level of antigenaemia per slide if CMV disease (mean or median)</th>
<th>Threshold considered high risk if reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gema (Baldanti et al. 1997)</td>
<td>52</td>
<td>&gt;100</td>
<td>Not reported</td>
</tr>
<tr>
<td>Francisi (Francisci et al. 1995)</td>
<td>49</td>
<td>59</td>
<td>Not reported</td>
</tr>
<tr>
<td>Salzberger</td>
<td>144</td>
<td>28</td>
<td>Not reported</td>
</tr>
<tr>
<td>Bek</td>
<td>144</td>
<td>Not reported</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Wetherill (Wetherill et al. 1996)</td>
<td>22</td>
<td>693</td>
<td>&gt;48</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of patients</th>
<th>Cut-off point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shinkai (Shinkai et al. 1997)</td>
<td>94</td>
<td>&gt;100 copies/ml of plasma</td>
<td>High predictive value for CMV disease</td>
</tr>
<tr>
<td>Rasmussen (Rasmussen et al. 1997)</td>
<td>75</td>
<td>&gt;32 copies/ 25ml of plasma</td>
<td>Associated with CMV retinitis</td>
</tr>
<tr>
<td>Bowen (Bowen et al. 1997)</td>
<td>97</td>
<td>&gt;0.25 log increase in baseline CMV DNA load (whole blood)</td>
<td>1.37-fold /0.25 log rise risk of CMV disease</td>
</tr>
<tr>
<td>Spector (Spector et al. 1998)</td>
<td>201</td>
<td>log increase in baseline CMV DNA load (plasma)</td>
<td>3.1-fold / log rise risk of CMV disease</td>
</tr>
</tbody>
</table>
3.3 Comparison of Nucleic acid hybridisation, culture, shell vial assay and polymerase chain reaction for the detection of CMV in the blood.

Hybrid Capture System (Digene Diagnostics, Silver Spring, MD), a solution hybridization assay, was compared to cell culture and shell via assay for the diagnosis of CMV infection, correlation with clinical disease, and response to therapy in 36 AIDS patients. Patients were divided into two categories and followed longitudinally: those with CMV disease, retinitis (n=19) or colitis (n=2), and controls (n=15) who exhibited no symptoms of retinitis at study entry but were CMV antibody positive and had <50 CD4 cells/mm³. Two hundred and thirty-six blood specimens were collected at 1 to 4 week intervals, between 1 and 29 specimens per patient. Consensus positives were determined by cell culture, shell vial assay, solution hybridization assay, clinical course, and response to therapy. The consensus was determined by either clinically active disease together with at least one test positive, or at least one assay positive on more than one subsequent sample, either with or without other assay becoming positive during follow up. A subgroup of 119 of these blood samples underwent qualitative PCR assay of CMV DNA.

3.3.1 Specimens.

EDTA blood and serum were collected at 1-4 wk intervals from AIDS patients who had CMV retinitis (19) or colitis (2) and controls (15) who exhibited no symptoms of retinitis at study entry but were CMV antibody-positive and had <50 CD4 cells/mm³. Blood was divided equally between culture and solution
hybridization assay (3.5 mls each), an aliquot was processed for PCR, and the serum was frozen.

3.3.2 Culture.

Leukocytes were separated from 3.5 mls blood (PMN-polymorphprep, Robbins Scientific Corp, Sunnyvale, CA) in a technique described Miller (Miller, 1992), resuspended in 2mls of Eagle's minimum essential medium and inoculated into two human foreskin fibroblast tubes (tissue culture, 0.5 mls each) and two human foreskin fibroblast vials (shell vial, 0.3 mls each) (Bartels, Inc, Issacuah, WA).

3.3.2.1 Cell Culture

For cell culture the inoculum was allowed to absorb for 2 hours at 37°C and was then replaced with fresh Eagle’s minimum essential medium. Tissue cultures were maintained for 4 weeks at 37°C and observed for the development of a typical CMV cytopathic effect. Cytomegalovirus strain AD-169 and the College of American Pathology CMV isolate (VR-2) proficiency testing standard were also grown of human foreskin fibroblasts and used as positive controls for all assays.

3.3.2.2 Shell vial assay

For shell vial assay each specimen was inoculated into one vial containing human foreskin fibroblasts grown on 12mm coverslips (Bartels, Issaquah, Wash.) as described by Gleaves. (Gleaves et al. 1984) Shell vial monolayers were fixed with acetone and stained for viral antigen, immediate-early and early (Chemicon, Temecula, CA) 2 days post-inoculation,(Gleaves et al. 1984) and examined for nuclear staining by indirect immunofluorescence assay. Briefly 150µl of the antibody
reagent was added to each shell vial, as per manufacturer instructions, incubated, and fluorescein isothiocyanate-conjugated (FITC) label added, washed in PBS and then allowed to dry. The entire monolayer was then examined at x200, with questionable areas viewed at x400.

Uninfected monolayers (negative controls) should show typical cellular morphology with no specific or only minimal non-specific fluorescence. Positive controls should reveal cells with intense (3-4+) apple green homogenous nuclear and some cytoplasmic fluorescence. A positive culture is one exhibiting characteristic specific staining of the nucleus and cytoplasm. An unsatisfactory culture is one that does not show characteristic specific fluorescence and exhibits either intense nonspecific florescence or excessive destruction of the monolayer by specimen toxicity or contamination.

3.3.3 Hybrid capture system

A detailed description of the method is given in section 3.5.4

Qualitative assay: Results are expressed as Positive, Negative, or Equivocal based on a calculated cutoff. The positive cutoff is the mean of the negative control x 2. Normalised results are obtained by the ratio: specimen RLU/positive cutoff value. Ratios ≥1.0 are considered positive, ratios <.75 negative, and ratios >75 and <1.0 are equivocal for DNA. Quantitative assay: results are expressed as pg CMV DNA/ml.

3.3.4 Polymerase Chain Reaction

DNA was extracted from 200μl of whole blood using the Isoquick extraction kit, according to manufacturer’s instructions (IsoQuick, ORCA Research Inc., Bothell,
Briefly, the whole blood was lysed with the lysis solution, and the manufacturer's extraction matrix was then added for the isolation of the template DNA. The DNA was subsequently precipitated by using isopropanol and 70% ethanolol and was resuspended in 200μl RNAase-free water, and 10μl used as template for amplification. Amplification reaction mixture consisted of sterile water, PCR buffer (Perkin-Elmer, Foster City, CA), 200μM each of dNTP, 2.5U AmpliTaq (Perkin-Elmer, Foster City, CA), 0.5pM CMV MIE 4B and 5 primers (sequences: MIE-5, CAG CAC CAT CCT CCT CTT CCT CTO G position 1165-1150, MIE-4B, Biotin-CCA AGC GGT CTC TGA TAA CCA AGC C, position 731-755, which produce a PCR product of 435bp), and template were combined in 100μl total volume. Amplification was performed on in a 9600 thermal cycler (Perkin-Elmer, Foster City, CA). Samples were heated to 94C for 5 minutes followed by 40 cycles consisting of denaturation (1min, 94C), primer annealing (30sec, 55C), primer extension (1 min, 72C), and 4C hold. The SHARP Signal system (Digene) was used to detect the 435bp biotinylated product, according to manufacturer's instructions. Briefly, the Digene SHARP Signal System is a sandwich capture molecular hybridization assay that utilises colourimetric detection. An aliquot of a PCR reaction containing 5'-biotinylated products is hybridised with a specific single-stranded RNA probe. The resultant RNA-DNA hybrids are captured through biotin onto the surface of streptavidin-coated microwells. Immobilised hybrids are then reacted with an anti-hybrid antibody conjugated to alkaline phosphatase and detected with a colourimetric substrate (PNPP). The intensity of the colour generated is proportional to the amount of biotinylated PCR product in each reaction.
A standard curve may be generated and the absorbance value of each specimen compared to the absorbance values of the controls to determine the concentration of PCR product in test reactions.

3.3.5 Results

A total of 236 specimens were obtained (range 1-29 samples/patient). A subgroup of 119 of 236 underwent PCR assay of CMV DNA in addition to the other assays. Nineteen specimens were equivocal by hybrid capture system and eliminated from the initial comparison (although these cases are discussed later in this section); comparison of cell culture and hybrid capture system for the remaining specimens (n=217) yielded the following results (see Table 3.2):

<table>
<thead>
<tr>
<th></th>
<th>Culture Positive</th>
<th>Culture Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive HCS</td>
<td>29</td>
<td>27</td>
<td>56</td>
</tr>
<tr>
<td>Negative HCS</td>
<td>2</td>
<td>159</td>
<td>161</td>
</tr>
<tr>
<td>Total HCS</td>
<td>31</td>
<td>186</td>
<td>217</td>
</tr>
</tbody>
</table>

Consensus positives were determined by cell culture, shell vial assay, hybrid capture, and clinical course. Cytomegalovirus was detected in 22/36 (61.1%) patient samples, 15/21 patients (71.4%) with retinitis/colitis and 7/15 (46.7%) controls, and in
60/236 (25.4%) specimens. Solution hybridization assay and cell culture/shell vial assay detected CMV in 56/58 (sensitivity, 96.6%) and 31/58 (sensitivity, 53.4%) positive specimens, respectively. (see Table 3.3)

Hybrid capture system-positive, cell culture-negative specimens (n=27): these were true positives and culture failures. These specimens were obtained from patients who were either subsequently cell culture-positive, had a series of hybrid capture system-positive results usually with increasing CMV DNA concentration, were not responding to antiviral therapy, were CMV PCR-positive, and/or who exhibited CMV disease.

There were 19 hybrid capture system-equivocal patients. These results could be categorised as follows: eleven of 19 patients were previously or subsequently hybrid capture system-positive; all were also PCR-positive and culture negative. One patient of 19 yielded a positive culture on the same day, the other had previous positive cultures. In 1/19 patients it was the only specimen obtained from this patient who developed fatal CMV encephalitis 1 month later, PCR was positive, culture was negative. One of 19 patients had been off antiviral therapy 1 week previously. Therapy was started and subsequent HCS results were negative. Four of 19 patients were culture-negative, and PCR was not available; 2 of these 4 patients had treated CMV retinitis. (see Table 3.4)

There were two hybrid capture system-negative, cell culture-positive specimens. Twelve specimens collected over 5 1/2 months, from a single study patient; were all hybrid capture system-negative. The cell culture-positive sample yielded 3 plaques at 17 days in tissue culture, was shell vial-negative, PCR-negative.
and although the patient had CMV retinitis at study entry, he was clinically responding to therapy. The patient’s retinitis remained inactive throughout the rest of the follow up period of 6 months. The other cell culture-positive, hybrid capture-negative specimen was obtained from a patient who expired from gram-negative sepsis. Tissue cultures yielded 1 plaque at 17 days, shell vial assay was negative, and PCR was negative.

**TABLE 3.3 Sensitivity – Excluding PCR data**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>(Number/Total) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrid capture</td>
<td>96.6</td>
<td>(56/58) x 100</td>
</tr>
<tr>
<td>Tissue Culture and/or Shell Vial</td>
<td>53.4</td>
<td>(31/58) x 100</td>
</tr>
<tr>
<td>Tissue Culture</td>
<td>46.6</td>
<td>(27/58) x 100</td>
</tr>
<tr>
<td>Shell Vial</td>
<td>15.5</td>
<td>(9/58) x 100</td>
</tr>
</tbody>
</table>

**TABLE 3.4 Comparison of Hybrid Capture (HCS) and Qualitative PCR**

<table>
<thead>
<tr>
<th></th>
<th>Negative HCS</th>
<th>Positive HCS</th>
<th>Equivocal HCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative PCR</td>
<td>55</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Positive PCR</td>
<td>17</td>
<td>36</td>
<td>5</td>
</tr>
</tbody>
</table>
3.3.6 Conclusions

Specificity for solution hybridization assay and cell culture /shell via assay, was 100%. Solution hybridization assay and cell culture / shell via assay correlated in identifying CMV-positive patients but solution hybridization assay detected more CMV positive specimens earlier in the course of infection in individual patients. Review of the clinical information suggests that solution hybridization assay has potential for detecting active infection and monitoring response to therapy. Solution hybridization assay is more rapid and sensitive than cell culture /shell via assay for detection of CMV in blood, yields objective quantitative results, but it is not as sensitive as qualitative PCR assay, although only a subgroup of 119 samples had PCR for CMV DNA performed.
3.4 Main Study: Quantitative CMV DNA levels in the blood and its relationship to CMV retinitis in patients with AIDS

We hypothesised that CMV DNA levels in peripheral blood leukocytes are associated with the development of active CMV retinitis.

Aims of the study

We sought to determine whether CMV DNA levels in peripheral blood leukocytes determined by quantitative solution hybridization assays might also be associated with the development of CMV disease. Furthermore, we sought to determine whether CMV DNA levels in patients with pre-existing CMV retinitis were related to reactivation of retinal lesions and to the development of extraocular CMV disease. If such relationships are found, treatment strategies might be developed that could prevent or treat reactivations in the earliest stages, thereby limiting additional tissue destruction. Tests for CMV in the blood might also identify patients in greatest need of systemically administered drugs in addition to, or in lieu of, local therapy for CMV retinitis.
3.5 Materials and Methods

3.5.1 Patients

Patients were enrolled in this study between August and November 1995 from the Center for Clinical AIDS Research and Education, UCLA School of Medicine, if they met the following criteria: HIV antibody-positivity, 18 years of age or older, CD4+ T-lymphocyte count less than 50/microliter, and CMV antibody-positivity. The study was conducted under an institutional review board-approved protocol with informed consent.

Two groups of patients were studied. The first group consisted of 10 patients who did not have active CMV disease at entry into the study, as determined by review of systems and examination, and had no history of clinically apparent CMV disease. The group included nine men and one woman. The woman had acquired HIV by blood transfusion; all of the men had acquired HIV sexually. The second group consisted of 11 patients who had CMV retinitis and no history of clinically apparent extraocular CMV disease. All were men who had acquired HIV sexually. All were being treated with cidofovir, foscarnet, ganciclovir, or a combination of these drugs. All drugs were received intravenously or orally; no patient had received intravitreous injections or implantation of ganciclovir intraocular devices. Patients had received between one and five (median three) previous courses of high-dose ("reinduction") therapy for disease reactivation prior to enrolment. Patients were on a variety of HAART regimes that were being introduced during the time of the study. The introduction of HAART regimes limited recruitment during the later part of the study period.
All patients were followed prospectively and evaluated every 2 to 4 weeks with examinations by an infectious disease specialist and an ophthalmologist; at each visit, blood was obtained for CMV DNA blood level and leukocyte count. With respect to patients receiving cidofovir, blood for CMV DNA blood levels was taken during the day preceding drug administration. Fundus photographs were taken at each visit and evaluated by a reader masked to CMV DNA blood levels using standard criteria. (Holland et al. 1995; Holland et al. 1989) The reader evaluated each set of photographs for the following factors: change in border opacity from the previous visit, movement of lesion borders from the most recent baseline visit (at diagnosis or first visit after most recent reinduction), and the development of new lesions. CMV retinitis lesions were considered to be active if they had persistent or increased amounts of border opacification since diagnosis or since the end of the last reinduction. In this study, progression was defined as the development of new lesions or any lesion border advancement; a threshold distance was not considered in evaluation of results. At each examination the following three factors were determined for each patient with CMV retinitis at baseline: (1) location of CMV disease (CMV retinitis vs. CMV retinitis and extraocular disease); (2) evidence of progression (progressed vs. not progressed); and (3) lesion activity (active vs inactive; for active lesions, patients were subcategorized on the basis of change since last examination as having increased, stable or decreased activity).

Changes in therapy were based on clinical evaluation by the treating ophthalmologist and not by CMV DNA blood levels.
3.5.2 Cytomegalovirus DNA Analysis

3.5.2.1 Method Summary

Cytomegalovirus DNA sequences in peripheral blood leukocytes were detected using a quantitative solution hybridization assay (Digene Hybrid Capture System, Digene Corporation, Beltsville, MD). Leukocytes were prepared as follows: 3.5ml EDTA-anticoagulated blood was suspended in lysis buffer, incubated for 15 minutes at 20-25 °C, and centrifuged at 1000 × g. Supernatant was discarded, and the leukocyte pellet was resuspended in sample diluent and denaturation agent, and incubated for 50 minutes at 70 °C. For hybridization, CMV probe mix was added and the sample further incubated for 120 minutes at 70 °C. The probe mix consisted of single-stranded RNA sequences complimentary to the IE1 and IE2 regions (26,900 base pairs), and the gp86 region (11,000 base pairs) of the CMV genome. Contents of the hybridization tubes were subsequently transferred to corresponding capture tubes coated with antibodies to the RNA:DNA backbone of RNA:DNA hybrids and incubated for 1 hour at 20-25 °C. Fluid was decanted; immobilised hybrids were reacted with an alkaline phosphatase conjugated antibody specific for the RNA:DNA hybrids; and capture tubes were incubated for 30 minutes at 20-25 °C. Following washing, chemiluminescent substrate (Lumiphos, Digene Corporation, Beltsville, NID) was added. After incubation for 30 minutes, tubes were placed in a luminometer and the emitted light was measured as relative light units (RLU). The intensity of the light emitted is proportional to the amount of target DNA in the specimen. A negative control and three positive standards were run in triplicate for each run. Positive standards had concentrations of complementary CMV DNA ranging from 16.6pg/ml
to 1660pg/ml (4 \times 10^5 \text{ genomes/ml to } 4 \times 10^7 \text{ genomes/ml}). A log-log plot of RLU versus concentration of complementary CMV DNA was generated and concentration of CMV DNA in pg/ml for patient samples was derived from the plot. Results were expressed in pg/ml and genomes/ml. The lower limit of accurate quantification is approximately 5 \times 10^3 \text{ genomes/ml (8pg/ml of CMV DNA)}, although the presence of CMV DNA at values lower than 5 \times 10^3 can be identified by the luminometer.

### 3.5.3 Method details

The Digene Hybrid Capture System is a solution hybridization antibody capture assay that utilises chemiluminescent detection. Specimens containing the target DNA hybridise with a specific CMV probe cocktail. The resultant RNA:DNA hybrids are captured onto the surface of a tube coated with antibodies specific for RNA:DNA hybrids. Immobilised hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for the RNA:DNA hybrids, and detected with a chemiluminescent substrate. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light, units (RLUs) on a luminometer. The intensity of the light emitted is proportional to the amount of target in the specimen.

The CMV DNA Assay may be performed to produce qualitative or quantitative results. In the qualitative format, an RLU measurement equal to or greater than the positive cut-off value indicates the presence of CMV DNA. An RLU measurement less than the positive cut-off value and greater than the equivocal cut-off value indicates an equivocal result. An RLU measurement less than the equivocal cut-
off value indicate the absence of CMV DNA sequences or CMV DNA levels below the detection limit of the assay. In the quantitative format, a calibration curve of the standards is plotted and the RLU value of each specimen is compared to the RLU values of the standards to determine the concentration of CMV DNA in test specimens.

The CMV probe contains a probe mixture specifically chosen to eliminate cross-reactivity with human or other herpes virus sequences (unpublished data from Digene Corporation, no cross reactivity has been found to herpes viruses and human DNA, the probe cocktail has been tested against HSV-1, HSV-2, EBV, VZV, HHV-6, at concentrations as high as $10^{10}$ viral particles/ml and against human DNA at concentrations as high as 200µg/ml). The CMV probe supplied with the Hybrid Capture CMV DNA assay is complementary to approximately 40,000 bp or 17% of the CMV genome (230,000 bp). When performing, assay in the quantitative format, the concentration of target detected (shown by the DCR-1 Luminometer output) can be used to determine the number of viral genomes in the volume of sample tested. A thorough discussion of this equivalence will be found in the Interpretation of Specimen Results section.

3.5.3.1 Specimen Collection and Handling

At least 7 mls of whole blood is collected in an EDTA tube. Collected blood must be processed within 48 hours from the time of collection. After 48 hours, adequate cell recovery may not occur. Immediately after collection, whole blood may be stored for up to 24 hours at 20-25 °C, and thereafter at between 2 and 8° C until processing.
1 X lysis buffer is removed from cold storage (2-8°C) immediately prior to use and 10 ml 1 X lysis buffer is added to labelled 15 ml conical tubes. 3.5 ml of whole blood is transferred to the conical tube containing the 1 X lysis buffer. This is then mixed thoroughly by inversion and incubated at 20-25°C for 15 minutes. The 15 ml conical tube is then centrifuged at 1000 x g for 15 minutes in a swinging bucket rotor. This will result in the white blood cells being pelleted. During centrifugation, the hybridization tubes are labelled. The supernatant is decanted, taking care not to disturb the cell pellet. The tubes are then placed upright in a rack to allow excess supernatant to drain from the walls to the bottom of the tube. Excess supernatant is removed with a transfer pipette taking care not to disturb the pellet. 1.5 ml of 1 X lysis buffer is then added to the cell pellet. The pellet is then resuspended by drawing the pellet and buffer up and down in a transfer pipette. All of the resuspended cell solution is transferred to a labelled specimen hybridization tube with a transfer pipette. The hybridization tubes are capped and incubated at 20-25°C for 10 minutes. The hybridization tubes are spun at 1000 x g for 15 minutes in a swinging bucket rotor or a variable speed microfuge. Supernatant is removed with a disposable transfer pipette, taking care not to disturb the cell pellet. A transfer pipette with an extra-fine tip is used to remove all remaining traces of the supernatant. The pellet is dislodged and resuspend by tapping tightly on the bottom of the tube or by gentle vortexing. If the specimen volume is greater than 80μl, then sufficient volume is transferred to another tube so that the remaining specimen has a volume of 50-80μl. The specimens (cell pellets) may be tested immediately in the CMV DNA assay or may be stored at -20°C for future testing.
Blood volumes between 1ml and 3.5ml may be processed and tested with this CMV DNA assay. However, sensitivity of CMV detection may be reduced due to the smaller sample size and the reduced amount of CMV DNA in the sample.

3.5.3.2 Specimen Preparation

Specimens and required reagents are removed from the refrigerator or freezer prior to beginning the assay, and allowed to reach 20-25°C, which takes about 15 to 30 minutes. Labelled specimen hybridization tubes containing cell pellets are then placed in a hybridization tube rack, in the same order in which they will be tested. A space is left in the rack for the detection reagent 2 (DR2) blank, negative control and the appropriate positive standards. These are tested first. The positive control as additional quality control, is tested in the middle of the test run. 75µl of sample diluent is pipetted into the bottom of each specimen hybridization tube using a repeating pipettor. Sample Diluent is not added to controls or standards. Care is taken not to touch the specimen or sides of the tubes, otherwise cross-contamination of specimens could occur. 50µl of denaturation reagent is pipetted into the hybridization tubes using a repeating pipettor. Care is again taken not to touch the pellet or sides of the tubes or cross-contamination of specimens could occur. Each hybridization tube is capped and tightened. Specimens should turn purple or greenish colour.

3.5.3.3 Standard and Control Preparation

The CMV DNA assay may be run in either a qualitative or quantitative format. In these studies quantitative assays were performed. The controls and standards or each type of assay should be as follows:
Standards and controls are tested in triplicate until satisfactory results have been obtained in several consecutive assays. Thereafter, negative control and positive standards 1, 2 and 3 are tested in duplicate for each batch of specimens tested. The positive control is tested in duplicate for each batch of specimens as described above. Specimens are tested once with CMV Probe.

The positive control contains a defined concentration of CMV DNA that is detectable using the CMV DNA assay. The labelled control and standard hybridization tubes are placed in the hybridization rack. 100µl of each control or standard is pipetted into the bottom of appropriately labelled hybridization tubes. A clean pipette tip is used for each transfer to avoid cross-contamination of standards and controls. 50µl of Denaturation Reagent is pipetted into the bottom of each control and standard hybridization tube using a repeating pipettor. Care is taken not to touch the tip of the pipette from one tube to the next to avoid cross contamination of the specimens. Standards and controls should turn dark purple. Each hybridization tube is then capped and tightened.

3.5.3.4 Denaturation and Hybridization

During the denaturation and hybridization steps, it should be checked that the water level in the waterbath is adequate to immerse the entire volume of specimen in the tube. As the CMV probe mix is viscous, care should be taken to ensure thorough mixing, and that the required amount is completely dispensed into each tube. Tubes that remain pink or purple after the probe and denatured specimen have been mixed have not received the proper amount of probe or have not been properly mixed.
Procedure

The hybridization tubes are placed in a rotary shaker set at 1100 rpm for 5 minutes to resuspend the cell pellets and ensure that reagents are well mixed. The hybridization tubes are then incubated in a 70°C water bath for 25 minutes. The hybridization rack is removed from water bath and visually inspected to check that the cell pellets have dissolved. Individual hybridization tubes are vortexed to break up and resuspend any remaining material. The entire contents (approximately 150μl) of each specimen hybridization tube are transferred to the bottom of a new labelled specimen hybridization tube using a micropipettor. Controls or standard tubes are not transferred. Care is taken not to let specimen adhere to the sides of the tube, and all of the specimen should be at the bottom of the tube. A clean pipette tip is used for each transfer to avoid cross-contamination of specimens. The purpose of the transfer step is to minimise false-positive or equivocal results that may be caused by undissolved orundenatured specimen adhering to the cap or the upper portion of the original hybridization tube. The hybridization tubes are covered with new caps. The hybridization rack is returned to the 70°C water bath, incubated for 25 minutes, and removed from water bath when the denaturation incubation is complete. All tube caps are then loosened a half turn; but not removed, and 50μl of CMV probe mix is pipetted into the bottom of each hybridization tube, using a repeating pipettor.

The caps on all hybridization tubes are then tightened and mixed thoroughly by vortexing each tube individually for 5 seconds or are placed in a hybridization rack and shaken on a rotary shaker set at 1100 rpm at 20-25°C for 5 minutes. A colour change (purple to yellow) should be noted to occur in each standard and
control tube. Specimens should turn brown to yellow. The tubes are then incubated in a 70°C water bath for 120 minutes.

3.5.3.5 Hybrid Capture

The hybridization tubes are removed from the water bath and allowed to cool for 5 minutes. The entire contents of the control, standard and specimen tubes are then transferred to the bottom of the corresponding capture tubes using 1ml transfer pipettes, using a new pipette for each transfer. The capture tubes are covered with a sheet of parafilm, and shaken on a rotary shaker set at 1100rpm, at 20°C for 60 minutes. The tubes are decanted when the capture step is completed, by inverting rack over sink and shaking out the contents with a downward motion. The tubes are then blotted by tapping firmly 2-3 times on clean absorbent paper.

3.5.3.6 Hybrid Detection

250μl of Detection Reagent is pipetted into each capture tube and covered with parafilm. The entire rack is shaken by hand several times side to side and back to front to mix. The rack is incubated at 20-25°C for 30 minutes.

3.5.3.7 Washing

The tubes are decanted by inverting rack over sink and shaking with a downward motion. The inverted tubes are blotted by tapping firmly 2-3 times on clean absorbent paper. Each capture tube is overfilled with diluted Wash Buffer. The Wash Buffer is decanted by inverting rack over sink and shaking with a downward motion, This process is repeated for a total of five washes. After the final wash, the tubes are shaken and inverted vigorously over a sink in order to remove excess Wash Buffer.
The capture tubes are drained on clean absorbent paper for 5 minutes and blotted thoroughly by tapping firmly several times on clean dry absorbent paper, as failure to drain tubes thoroughly may result in a decreased signal. To prevent contamination the tubes are inverted or covered until Detection Reagent 2 is added.

3.5.3.8 Signal Generation

250 µl of Detection Reagent 2 is pipetted into an empty clean polystyrene tube. 250µl of Detection Reagent 2 equilibrated to 25°C is pipetted into each capture tube. The tubes are covered with clean parafilm, and incubated at 25°C for 30 minutes, avoiding direct sunlight. The bottom of each capture tube is gently wiped with a damp Kimwipe and read on a luminometer (DCR-1 Luminometer). All tubes are read immediately after the 30 minute incubation is completed.

If specimen tubes remain to be read after 5 minutes has elapsed, the Negative control and the positive standards are reread in the luminometer again in order to establish a new cut-off value to measure the results of the remaining test run accurately. This process is repeated every 5 minutes until all specimen tubes have been tested.

3.5.3.9 Quality Control

As a validation procedure, positive standards are tested in triplicate until satisfactory results have been obtained in several consecutive assays. For a quantitative assay, the negative control and positive standards 1, 2, and 3 are tested in duplicate for each test run after successful validation testing of controls and standards in triplicate. The Detection Reagent 2 blank should have an RLU value < 5000 and
should be less than all control values. Values above 5000 RLUs suggest Detection
Reagent 2 contamination, and specimen results cannot be interpreted in this case. All
control results should demonstrate variability < 30%. Variability is estimated from a
number of replicate measurements of a given sample. The equation used to estimate
the variability (expressed as a percentage of the mean) is:

$$\sqrt{\frac{\sum (x_i - \bar{x})^2 / n}{\bar{x}}} \times 100$$

If the variability of any triplicate result is > 30%, the control value with a RLU
value furthest from the mean is discarded as an outlier and the mean is recalculated
using the remaining two control values. If the recalculated variability is < 30%, the
next step of the calculation is proceeded to; otherwise, the assay is invalid and is
repeated. The mean of the Negative control results should be <12,000 RLUs. If the
mean of the negative control is >12,000 RLUs, the assay is invalid and must be
repeated. The positive standard mean (PSx) and negative control mean (NCx) results
are used to calculate the ratios that validate the assay. These results must be within
the given acceptable ranges, for the DC-1 Luminometer to validate the assay before
the specimen results can be interpreted (see Table 3.5):

### Table 3.5 Cutoff Calculation

<table>
<thead>
<tr>
<th>Assay Validation Acceptable Ranges (for DCR-1 Luminometer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative Assay</td>
</tr>
<tr>
<td>10 ≥ PS1x / NCx ≥ 1.5</td>
</tr>
<tr>
<td>100 ≥ PS2x / NCx ≥ 10.0</td>
</tr>
<tr>
<td>800 ≥ PS3x / NCx ≥ 80.0</td>
</tr>
</tbody>
</table>


The appropriate ratio(s) shown above is calculated using the mean values on the luminometer printout tape. If any of the calculated values fall outside its acceptable range, the assay is invalid and is repeated.

The positive control contains a defined concentration of CMV DNA that is detectable using the CMV DNA assay. This sample may be tested to meet the quality control requirements of the testing laboratory. Acceptable values for the positive control are: 15-28 pg/ml for the quantitative assay.

A CMV DNA test panel (Digene Diagnostics Inc, Catalog No. 4403-1004) consists of six samples, one negative and five positives which have defined concentrations of CMV DNA that are detectable using the CMV DNA assay. These samples may be used for quality control of each run or may be tested over defined time intervals for quality assurance purposes.

Cutoff calculation: the positive CMV cutoff value is taken as mean \([NCx] \times [2]^3\)

\[
\begin{array}{ll}
\text{NC RLU Values} & \text{PS1 RLU Values} \\
3393 & 9492 \\
3336 & 10419 \\
3385 & 9750 \\
\end{array}
\]

Variability 0.7% 3.9%
Mean Value 3371 9887
PS1x/NCx N/A 2.9

Assay is valid therefore, Positive Cutoff Value = \([3371] \times [2] = 6742\)
3.5.3.10 Interpretation of Specimen Results

Specimens with RLU values ≥ positive cutoff value are considered ‘Positive’ for CMV DNA. Specimens with RLU values < positive cutoff value and > 90% of the positive cutoff value are considered ‘equivocal’ for CMV DNA. A second sample should be obtained and tested. Specimens with RLU values < 90% of the positive cutoff value contain CMV DNA levels below the detection limit of the assay or do not contain CMV DNA. These are interpreted as ‘No CMV DNA detected’.

When performing the quantitative assay, only specimens with RLU values between positive standard 1 and positive standard 3 RLU values can be quantified accurately since this is the linear range of the assay.

Table 3.6 CMV Genome Equivalents of Complementary DNA

<table>
<thead>
<tr>
<th>CMV Genome Equivalents Complementary DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target genomes/ml</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Negative Control</td>
</tr>
<tr>
<td>CMV Positive Standard 1</td>
</tr>
<tr>
<td>CMV Positive Standard 2</td>
</tr>
<tr>
<td>CMV Positive Standard 3</td>
</tr>
</tbody>
</table>

To determine the actual concentration of CMV in the specimen, the number of genome equivalents detected must be calculated from the concentration of complementary DNA reported by the luminometer using the following equation that relates the concentration of DNA detected to the number of CMV genome equivalents (16.6 pg/ml = $4 \times 10^4$ genomes/assay) (see Table 3.6):
CMV genomes per assay = reported CMV DNA Concentration (pg/ml) x 2410 genomes-ml/assay-pg

3.5.4 Data Analysis

CMV DNA levels were expressed both as genomes per millilitre of blood and as genomes per leukocyte, because fluctuating leukocyte levels might affect results. In addition to examination of changing CMV DNA values over time, a single median and mean CMV DNA value was calculated for each patient using values from all visits. For the purposes of this study, stated median values were determined using all the quantitative data, even if levels were below the limit of accurate quantitation. Undetectable levels were assigned a value of zero for purposes of analysis. Values for CMV DNA below the limit of quantification are indicated in the figures but were not assumed to be accurate.

CMV DNA blood levels for individual patients were compared to changes in clinical factors over time. Also, the distributions of all individual CMV DNA blood level measurements were compared after grouping values on the basis of the various patient factors present at the time each measurement was determined.

The software used for statistical analysis was SPSS for Windows Release 6.1.

3.5.5 Results

Patients without CMV Disease at Baseline

A summary of patient data is listed in Table 3.7. The median length of follow-up for this group was 16 weeks and the median number of evaluations was six. Patients fell into two distinct groups: those whose CMV DNA levels stayed

93
persistently below 5000 genome/ml (n=6), and those having levels above this value at some point during follow-up (n=4). In three of four patients with levels above 5000 genomes/ml CMV retinitis developed during the follow-up period. The one patient that did not develop CMV retinitis had signs and symptoms consistent with CMV oesophagitis, although endoscopic biopsy did not confirm infection. This patient was treated with ganciclovir at the onset of oesophageal symptoms, at which time examination of the fundus revealed no signs of CMV retinitis. A post hoc analysis revealed a strong association between development of CMV disease and elevation of CMV DNA levels in the blood above 5000 genomes/ml. While each of the four individuals who achieved levels greater than 5000 genomes/ml during the course of follow-up developed CMV disease (3 cases of CMV retinitis, one case of extraocular disease), none of the six individuals with CMV DNA blood levels consistently below 5000 genomes/ml developed CMV disease (p=0.05, Fisher exact test).

Patients who developed CMV disease had a mean CMV DNA blood level of 6676 genomes/ml, a median of 2920 genomes/ml, and a range of below detectable limit to 55,624 genomes/ml, during the follow up period up to the time of clinical onset of disease. (see Figure 3.2a)

Patients who never developed CMV disease during the study had a mean CMV DNA level of 917 genomes/ml, a median below detectable limits and a range of below detectable limit to 1590 genomes/ml during the follow-up period (see Figure 3.2b).
Table 3.7 Patients without Clinically Apparent CMV Disease at Baseline

<table>
<thead>
<tr>
<th>Patient Numbers</th>
<th>Number of follow-up visits</th>
<th>CMV DNA levels in the blood (genomes/ml)</th>
<th>Development of CMV retinitis</th>
<th>Development of Extraocular disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial level</td>
<td>Median level*</td>
<td>Pattern of change+</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>31,110</td>
<td>3,222</td>
<td>fluctuating</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>11,995</td>
<td>6,206</td>
<td>decreasing</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>786</td>
<td>2,772</td>
<td>fluctuating</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>1,501</td>
<td>undetectable</td>
<td>fluctuating</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>1,434</td>
<td>undetectable</td>
<td>decreasing</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>undetectable</td>
<td>undetectable</td>
<td>fluctuating</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
</tbody>
</table>

* Median of all values taken between baseline and development of CMV disease.

+ "Fluctuating" defined as either CMV DNA levels changing from undetectable to greater than 5000 genomes/ml and back to undetectable levels, or changing from greater than 5000 genomes/ml to undetectable and then back to greater than 5000 genomes/ml.
Figure 3.2

Changes in CMV DNA blood levels over time in patients with no CMV disease at baseline. Graph A describes three patients who developed CMV retinitis and one patient who developed presumptive CMV oesophagitis during follow-up. Points have been connected to facilitate identification of data for a given patient, but because of the fluctuating nature of CMV DNA blood levels and because examinations were intermittent, the line does not necessarily reflect the true level at any given time point; thus, CMV DNA level were not necessarily falling when CMV retinitis developed. Graph B describes the six patients that did not develop CMV disease during follow-up. Most patients had unrecordably low CMV DNA blood levels during follow-up. Values below about 5000 genomes/ml cannot be quantified accurately (shaded region).
Patients with CMV Retinitis at Baseline

A summary of patient data is listed in Table 3.8. During the course of the study, this group of patients had a mean CMV DNA blood level of 147,084 genomes/ml, a median of 4500 genomes/ml, with a range of below detectable limit to 2,685,428 genomes/ml. This group was followed for a median of 16 weeks, and the median number of evaluations was seven. Activity of CMV retinitis lesions was characterised during the study as follows: four with persistent inactivity; three with varying degrees of activity and inactivity; and four with persistent activity.

The patients with persistent activity had had multiple previous reinductions (median three) with various therapies.

The distributions of all individual CMV DNA blood level determinations are shown in in Figure 3.3 after grouping values on the basis of various patient factors present at the time each measure was determined. The median CMV DNA blood level for examinations at which CMV retinitis was the only clinically apparent manifestation of CMV disease was lower (median 1807 genomes/ml) than for those examinations at which extraocular CMV disease was also present (median 170,239 genomes/ml), although the distributions overlap (Figure 3.3a).

Values above the median tended to be those measured in patients who eventually developed clinically symptomatic extraocular disease. Outlier and extreme values among patients with only CMV retinitis were obtained from patients who had active retinal disease or disease that reactivated shortly thereafter.
Table 3.8  Patients with CMV Retinitis at Baseline

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Number of follow up visits</th>
<th>CMV DNA levels in blood (genomes/ml)</th>
<th>CMV retinitis activity</th>
<th>Development of Extraocular Disease (time developed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial level</td>
<td>Median level*</td>
<td>Pattern of change</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>undetectable</td>
<td>24,782</td>
<td>increasing</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>undetectable</td>
<td>147,845</td>
<td>increasing</td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>302,834</td>
<td>160,283</td>
<td>fluctuating+</td>
</tr>
<tr>
<td>14</td>
<td>18</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>2,939</td>
<td>136,130</td>
<td>fluctuating+</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>252</td>
<td>42,864</td>
<td>increasing</td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
<tr>
<td>18</td>
<td>8</td>
<td>4,658</td>
<td>12,945</td>
<td>fluctuating+</td>
</tr>
<tr>
<td>19</td>
<td>8</td>
<td>undetectable</td>
<td>undetectable</td>
<td>stable</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>undetectable</td>
<td>3,009</td>
<td>fluctuating+</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>4,708</td>
<td>26,431</td>
<td>increasing</td>
</tr>
</tbody>
</table>

*Median of all values taken between baseline and development of active CMV disease.

+“Fluctuating” pattern of change defined as either CMV DNA levels changing from undetectable to greater than 5000 genomes/ml and back to undetectable levels, or changing from greater than 5000 genomes/ml to undetectable and then back to greater than 5000 genomes/ml.

++ Fluctuating activity defined as having a change from active to inactive or vice versa at some point during course of the study.
Boxplots showing distribution of all individual CMV DNA blood level determination in patients with CMV retinitis at baseline, categorised at the time of each determination by location of CMV disease (Figure 3.3a), evidence of progression (3.3b), and activity of retinal lesions (3.3c). For each boxplot, the central dotted line represents the median value of CMV DNA blood level determinations in that category; the box covers the inter-quartile range; and the lines encompass the 23-97.5 percentiles. Outlying values (values more than 1.5 box-lengths from the 25th or 75th percentile) are represented by open circles; extreme values (values more than 3 boxlengths from the 25th or 75th percentile) are represented by asterisks. Outlier and extreme values in the inactive disease category (Figure 3.3c) occurred in patients who subsequently developed either reactivation of CMV retinitis or development of extraocular disease within an 8-week period.
The median CMV DNA blood level was higher for measurements taken when progression was identified (167,598 genomes/ml) than for those measurements taken when no progression was identified (1117 genomes/ml) (Figure 3.3b).

The outlier and extreme values among patients that did not have progression were in patients with either active CMV retinitis or those that developed extraocular disease shortly thereafter.

Median values were higher and ranges were larger for CMV DNA blood levels that were determined when disease was active, although distributions overlapped. (Figure 3.3c). Outlier values among patients with inactive disease were obtained either from patients whose CMV retinitis reactivated within 7 weeks, or in the case of very high values, who developed extraocular disease.

Serial time graphs displaying data for selected patients are shown in Figures 3.4, 3.5, and 3.6. Of the four patients with persistent inactivity of CMV retinitis throughout the course of study, only one (patient 16) developed CMV DNA levels greater than 5000 genomes/ml; this patient had an increase in levels by a factor of $10^5$ during the course of follow-up (Figure 3.4). He developed CMV colitis, which was confirmed by endoscopy at week 8. The three with low or undetectable levels of CMV DNA throughout the study period did not develop extraocular disease during their 20 weeks of follow-up.

Each of the four patients with persistently active CMV retinitis during the study developed extraocular CMV disease. Figure 3.5 describes data from patient 12 who had persistent activity throughout the course of the study. He had failed multiple
previous courses of therapy, and was receiving both foscamet and ganciclovir.

Following progression of CMV retinitis at week 3, he was reinduced with cidofovir. Despite this change in therapy, values continued to rise. At week 10, he developed new gastrointestinal symptoms, and was found to have CMV colitis by endoscopy at week 12. He died at week 14 and autopsy revealed CMV encephalitis and colitis.

In the three patients with fluctuating CMV retinitis activity during follow-up, three of four reactivations were preceded by at least a tenfold rise in CMV DNA blood levels, occurring 2 to 6 weeks before reactivation was detected clinically. In all three patients, CMV retinitis became inactive with reinduction; this change was accompanied by at least a 10-fold reduction of CMV DNA blood levels from that measured at the start of reactivation. None of the patients in this group developed extraocular CMV disease. Figure 3.6 shows the DNA levels over time for patient 18, who had fluctuating activity. He finished a course of reinduction just before the start of the study. The DNA levels were falling and then promptly rose again between weeks 4 and 6; CMV retinitis reactivated by week 10 at which time CMV DNA levels were very high. He was reinduced and CMV DNA levels fell as the disease became inactive by week 12.

All patients with CMV retinitis at baseline who had a 10-fold or greater rise in CMV DNA blood levels developed extraocular CMV disease, reactivation of CMV retinitis, or both during follow-up. Conversely, some of the patients with reactivation of CMV retinitis had low/undetectable levels of CMV DNA. In contrast, all five patients that developed extraocular disease did have high and rising (greater than 10-fold between successive visits) blood levels of CMV DNA before the onset of the extraocular disease.
Normality of variable data (only the median value of each patient analysed) was assessed by the Shapiro-Wilks Test. The leukocyte count and the natural log transform of genomes/ml approximated a normal distribution. There was a significant correlation between leukocyte count and CMV DNA blood levels measured in genome/ml ($r = 0.438$, $p = 0.045$). There was also a significant relationship found between CMV DNA blood levels expressed as genomes/ml and genomes/leukocyte (Pearson correlation coefficient, $r = 0.907$, $p < 0.001$).
Figure 3.4

Changes in CMV DNA blood levels over time for four patients with clinically inactive CMV retinitis. Patient 16 was the only patient with consistently rising CMV DNA levels and the only patient to develop extraocular CMV disease; colitis was diagnosed at week 10, although he had symptoms of colitis by week 8. Values expressed in genomes/leukocyte and those expressed in genomes/ml are shown on the same graph only to demonstrate that the time course of changes is similar; scales on the y-axis are different. Values for genomes/leukocyte were not calculated when CMV DNA level determinations fell below the level of accurate quantification; hence the plot for Patient 17 is discontinuous. For the graph showing values in genomes/ml, values in the shaded region cannot be quantitated accurately.
Figure 3.5

Changes in CMV DNA blood levels over time in patient 12, who had persistent clinical activity of CMV retinitis during the study period. He had rising CMV DNA blood levels, expressed both in genomes/ml and in genomes/leukocyte, despite reinduction with cidofovir at week 3, following progression of disease. The CMV lesions became less active, but CMV DNA blood levels continued to rise despite therapy. At week 10 the patient developed CMV colitis followed by CMV encephalitis at week 15. The patient died at week 17. Values expressed in genomes/leukocyte and those expressed in genomes/ml are shown on the same graph only to demonstrate that the time course of changes is similar; scales on the y-axis are different. Values for genomes/leukocyte were not calculated when CMV DNA level determinations fell below the level of accurate quantification. With regard to values in genomes/ml, those in the shaded region cannot be quantitated accurately.
Changes in CMV DNA blood levels over time in patient 18, who had fluctuating clinical activity of CMV retinitis during the study period. He received cidofovir induction therapy (5mg/kg for 2 weeks) beginning at week 0, because of disease progression while receiving intravenous ganciclovir therapy. The retinitis became inactive and the CMV DNA blood levels became undetectable by the time of the patient’s next visit. At week 4, the cidofovir dose was reduced (3mg/kg) because of renal insufficiency. Cidofovir therapy was then stopped, and CMV DNA blood levels began to rise between weeks 5 and 10. CMV retinitis reactivated at week 10, and reinduction using ganciclovir resulted in decreased clinical activity of CMV retinitis and decreased CMV DNA blood levels. The patient’s leukocyte count was boosted at week 4 by administration of filgrastim. Values expressed in genomes/leukocyte and those expressed in genomes/ml are shown on the same graph only to demonstrate that the time course of changes is similar; scales on the y-axis are different. Values for genomes/leukocyte were not calculated when CMV DNA level determinations fell below the level of accurate quantification, with regard to the values in genomes/ml, those in the shaded region cannot be quantitated accurately.
3.5.6 Discussion

In HIV-infected individuals, quantitative measures of HIV load are being used with increasing frequency to monitor the status of HIV infection for the purpose of determining prognosis and aiding in the choice of therapy. (Merigan, 1995; Harrigan, 1995) It may be that quantitative measures of CMV load may also be useful for following the course of CMV disease and its response to therapy in HIV-infected patients. Despite the fact that the incidence of CMV retinitis has dropped in association with protease inhibitor use, there is still a need for such techniques; it could be argued that it is even more important now to have better tools that will identify the specific, smaller subset of patients still at risk for CMV disease and its complications, thereby avoiding unnecessary use for expensive and toxic anti-CMV drugs in the larger population.

In solid organ and bone marrow transplant recipients, identification of pre-clinical CMV disease has been attempted through detection of CMV DNA in peripheral blood, (Wolf and Spector, 1993; Saltzman et al. 1992) because preemptive therapy has been found to be associated with a better outcome than prophylactic therapy with respect to both morbidity and mortality in this population. (Goodrich et al. 1993; Goodrich et al. 1991; Singh et al. 1994) The ability to detect CMV disease in a pre-clinical disease state may allow similar strategies to be applied with HIV-infected patients. In untreated HIV-infected patients with CMV disease, CMV blood levels are known to increase with time and a high virus load is associated with the development of extraocular disease. (Bowen et al. 1995)
Urine and blood cultures, the established tests for diagnosing replicative CMV infection, are of low positive- and negative-predictive value for the development of CMV disease in HIV infected patients. (Salmon et al. 1990; Zurlo et al. 1993; MacGregor et al. 1995) The CMV antigenemia assay is a quantitative test that uses monoclonal antibodies to detect the CMV pp65 protein; this assay has been shown to have potentially good predictive value for determining those HIV-infected patients who will develop CMV disease, (Francisci et al. 1995; Wetherill et al. 1996; Revello et al. 1992; Erice et al. 1992) but is labor-intensive, subjective with respect to interpretation of test results, and requires evaluation of samples within 6 hours of collection, since delay can result in a substantial decrease in the observed levels of positivity. (Landry et al. 1995; Boeckh et al. 1994; Gerna et al. 1991) PCR-based amplification assays for detecting viral DNA in blood components have also been used for diagnosing established CMV infection and have been investigated as a possible test for predicting which patients will develop CMV disease. (Hansen et al. 1994; Bowen et al. 1997; Dodt et al. 1997; Spector et al. 1998; Drouet et al. 1993; Rasmussen et al. 1995; Shibata et al. 1988; Spector et al. 1992) As with the antigenemia assay, initial studies suggest PCR techniques have a high positive predictive value for development of CMV disease. Disappearance of leukocyte CMV PCR positivity has also been used as a measure of therapeutic response. (Gerna et al. 1994) There are a number of problems with PCR-based quantification techniques, however. The high sensitivity of this technique may result in the detection of considerably more patients with asymptomatic infection than culture or antigen detection. In addition, the test is prone to false-positive results because of contamination; it is more labour-intensive than other tests; and errors may arise in
quantitation, due either to the variable efficiency of the control reaction or inability to
detect PCR inhibitors.

Assays based on signal amplification, such as the quantitative solution
hybridization assay used in this study, are easy to perform, rapid (taking about 6 hours
to complete), are potentially of lower cost than PCR techniques (based on the rapidity
of the technique and the lack of technical skill and equipment necessary to perform
it), and can produce quantitative CMV assays on blood leukocytes. These properties
make the solution hybridization test applicable to routine clinical use. The CMV
solution hybridization assay has been reported to be a reliable test for the detection
and quantification of CMV DNA in the blood (T. Mazzuli and C. Isada, unpublished
data presented at The 3rd Conference on Retroviruses and Opportunistic Infections,
Washington DC, 1996). It has a sensitivity greater than shell vial assay but less than
that of PCR-based assays (M. Miller, unpublished data presented at the American
Society for Microbiology Annual Meeting, New Orleans, LA 1996); the lower limit of
accurate quantitative detection is approximately 5000 genomes/ml. (Imbert-Marcille
et al. 1995)

A problem to be addressed with virus load assays is whether differences exist
in the amount of CMV DNA in the leukocytes and plasma, and if so, which pool most
closely reflects disease activity. CMV is both latent in, and has its early replicative
phase in, leukocytes; (Gema et al. 1992; Taylor-Wiedeman et al. 1991) therefore,
changes in CMV DNA levels may take place earlier in leukocytes than in the plasma.
Alternatively, if the CMV blood levels reflect replication in a particular organ, then
plasma levels, derived from virus being shed from that organ, may reflect changes in
body load better than leukocyte levels. Leukocyte levels fluctuate commonly in
patients with HIV disease, which might affect reliability of results. The leukocyte CMV DNA level is known to be related to the plasma level, implying that it may not be important which level is measured clinically. (Zipeto et al. 1995) In this study it appears that the level of CMV DNA obtained in genomes/ml approximately parallels the change in levels of genomes/leukocyte (Figures 3.4 to 3.6), implying that fluctuating leukocyte counts are probably not detrimental to the predictive value of the test.

The lower sensitivity of the Digene Hybrid Capture System for detection of CMV DNA in comparison to PCR-based techniques may, in fact, have some advantages. Lower sensitivity may avoid detection of low levels of CMV DNA that could be of questionable clinical importance. Nevertheless, the greater sensitivity of PCR-based techniques over a solution hybridization assay may allow for the detection of increasing CMV DNA blood levels at an earlier stage. Attempts at early detection by more sensitive assays may, however, be prevented by low level fluctuation in CMV DNA blood levels; in patients with clinically stable CMV retinitis and without extraocular disease, there is low level undulation of CMV DNA blood levels that may be due in part to variability of the assay and in part to actual fluctuations in CMV DNA blood levels. The Digene Hybrid Capture System has been found to have good intra- and interrun precision. (Mazzulli et al. 1996)

The predictive value of the test may be dependent on the frequency of test repetition, because of the undulation of CMV DNA levels before the development of CMV disease. Performing virus load assays too infrequently may identify values in a "trough" of the undulations and give a false negative result. Further study is needed to
establish the optimum frequency with which virus load assays are performed to maximise their predictive value.

Our results support other studies suggesting that rising blood levels of CMV in HIV-infected patients indicates increased risk for development of CMV disease and suggests that quantitative solution hybridization assays may be another effective technique for making such measurements. There was a statistically significant association between CMV DNA blood levels that rose above 5000 genomes/ml and development of CMV disease, although this observation must be viewed with caution; the cut-off of 5000 genomes/ml was not chosen apriori, and such post hoc analyses can present a selection bias, with a distorted picture of prognostic factors. (Saltzman et al. 1992) The association, if true, may allow preemptive therapies to be developed that will prevent CMV disease, while sparing the expense and potential toxicity of prophylactic drug for the 60% to 75% of patients that would never develop CMV disease.

In this pilot study, our observations also suggest that relationships can be identified between quantitative measures of CMV DNA in the blood and subsequent changes in the activity of pre-existing CMV retinitis lesions in some patients, although sample sizes were too small to confirm these relationships in any type of statistical analysis or to establish the predictive values of various CMV DNA blood levels. Patients with extraocular CMV disease also appeared to have higher CMV DNA blood levels than patients with CMV retinitis alone, which supports previous findings by Saltzman and associates. (Saltzman et al. 1992) Although there appeared to be a general relationship between the severity of disease and CMV DNA blood levels, active CMV retinitis could also occur with low blood levels of CMV DNA,
even when reinduction therapy was not initiated. The fact that a range of CMV DNA blood levels is observed with active CMV retinitis may be explained by varying degrees of peripheral CMV replication. Replication may be limited to the eye in some patients, due to a variety of factors such as the occurrence of local resistance, inadequate intraocular drug levels, or recurrence of disease activity earlier in the eye than elsewhere. No relationship was seen between the type of systemic therapy used and CMV DNA blood levels in this study. All drug therapies used in this study were able to lower CMV DNA blood levels to below quantifiable levels for the solution hybridization assay used.

Increasing CMV DNA levels in patients with retinal disease was followed by reactivation of retinitis or development of extraocular CMV disease in all cases. In all such patients, retinal opacity was seen along broad and extensive segments of pre-existing borders, indicating that reactivation of virus already present in preexisting lesions was responsible for recurrence of active CMV retinitis, rather than development of new disease foci from circulating CMV in the bloodstream. The association probably reflects waning immunity or inadequate drug levels throughout the body that allows reactivation from multiple sites, including the eye. The SOCA Research Group (Studies of Ocular Complications of AIDS (SOCA) Research Group in collaboration with the AIDS Clinical Trials Group (ACTG), 1998) found that CMV-positive blood cultures at baseline are associated with more rapid times to disease progression; progression was based on advancement of pre-existing lesion borders, rather than on the development of new lesions. While this observation does not confirm a relationship between blood levels of CMV and reactivation of CMV
retinitis, it does support the fact that relationship can exist between vireamia and local, intraocular phenomena.

Additional studies may indicate a role for CMV DNA blood levels in planning preemptive therapy before changes in CMV retinitis lesions become apparent clinically. Current therapeutic strategies rely on the clinical observation of lesion reactivation or appearance of new lesions as a trigger for alteration of therapy. Preemptive therapy that prevented an anticipated reactivation would avoid the additional tissue destruction that occurs with reactivation and progression. Reactivation of CMV retinitis was not always associated with rising CMV DNA levels, however. Determination of CMV load may therefore have important implications for the choice between systemic and local therapy. Reactivation with low levels of CMV in the blood may imply either inadequate ocular drug levels or localized virus resistance, and supplemental local therapy alone may be sufficient to bring disease under control. On the other hand, high CMV levels in the blood may reflect reactivation of disease throughout the body that requires systemic therapy. Additional study of outcomes will be required before choice of local versus systemic reinduction therapy can be based on the results of virus load tests, but our observations suggest that it is reasonable to expect that eventually such choices might be possible. Also, the failure to detect rising levels of CMV DNA before reactivation of CMV retinitis in some cases shows that CMV DNA levels alone cannot be used for determining the need to change therapy, and that regular examinations by an ophthalmologist are still necessary.

We did not study patients receiving local therapy, but CMV load could also play a role in monitoring such patients. Changes in blood levels of CMV DNA would
be expected to have less relationship to reactivation of CMV retinitis in such cases, but could help to identify a subgroup of patients who are at risk for development of non-ocular CMV diseases or development of fellow eye involvement and therefore might benefit from additional systemic therapy.
Chapter 4 Haemorheologic Abnormalities

4.0 Introduction

Retinal microvasculopathy is common in individuals with AIDS, being present in greater than 43% patients in the pre-HAART era. (Newsome et al. 1984; Pepose et al. 1985; Freeman et al. 1989) The predominant clinical manifestations of this microvasculopathy are cotton-wool spots (see Figure 4.1). They are more common in patients with AIDS than in HIV-infected individuals without AIDS, and are more frequently observed before the development of CMV retinitis. (Jabs, 1995) Other clinical manifestations include retinal haemorrhages, microaneurysms, abnormal haemodynamics at the level of the perifoveal capillaries (as measured by scanning laser ophthalmoscopy) (Yung et al. 1996) and microvascular abnormalities on fluorescein angiography. (Pepose et al. 1985; Newsome et al. 1984) Ultrastructural studies of retinal capillaries demonstrate basal lamina thickening, swelling of endothelial cells, narrowing and occlusion of vascular lumina, and loss and degeneration of pericytes. (Newsome et al. 1984; Pepose et al. 1985) It has been hypothesised that microvascular disease may facilitate CMV entry to retinal tissue through a damaged blood retinal barrier. (Pepose et al. 1985)

Rheologic abnormalities, including markedly increased red blood cell aggregation and plasma viscosity, have been documented in HIV infected individuals. (Engstrom et al. 1990) The presence of cotton wool spots, a sign of retinal microvasculopathy, have been associated with the development of CMV retinitis. (Jabs, 1995) Rheologic abnormalities may contribute to both vascular endothelial damage (Engstrom et al. 1990) and sludging of blood which may
modulate the CMV load in retinal vessels. Although Engstrom and associates (Engstrom et al. 1990) have described haemorheologic abnormalities in HIV-infected individuals it has not been shown whether there is a difference in factors that may affect blood flow in those HIV infected patients with CMV retinitis compared to those without.

Leukocytes are larger and more rigid than erythrocytes (Moessmer and Meiselman, 1990) and have been shown to have a strong influence on microvascular blood flow (Sutton and Schmid-Schonbein, 1992). In addition, polymorphonuclear leukocytes (PMN), which comprise the largest fraction of leukocytes, are capable of causing direct microvascular damage by the release of proteases and toxic oxygen radicals (Weiss, 1989). Leukocytes are probably the main reservoir for latent CMV, (Lang et al. 1977; Cassol et al. 1989; Stanier et al. 1989)

**Hypothesis**

We hypothesise that the combination of an effective localised increase in CMV load in retinal vessels, due to decreased flow, and microvascular endothelial damage may increase the risk of the development of CMV retinitis. Altered rheological properties of leukocytes and changes in blood constituents, that may alter flow in the retinal vessels, may therefore contribute to the risk for the development of CMV retinitis.
Multiple cotton-wool spots, associated with the presence of the retinal microvasculopathy of HIV-infection.
Aim

We undertook to explore whether there is a difference in factors that may affect blood flow in those HIV infected patients with CMV retinitis compared to those without. Little is known about the rheological behaviour of leukocytes from such subjects and we therefore specifically also undertook to explore the relationship between PMN rigidity and CMV retinitis in HIV-infected individuals.

4.1 Principles of Clinical Blood Rheology

4.1.1 Introduction

A liquid flows in a tube along an energy gradient. As it flows from a higher energy point to a lower energy point, energy is dissipated in overcoming frictional losses both within the fluid, and between the fluid and the tube wall. Resistance to flow is partly geometric resistance imposed by the tube, and partly viscous resistance between particles in the flowing blood.

The relationship between volume rate, streamline flow in a straight, rigid tube depends on the pressure gradient along the tube, the length and radius of the tube; and the viscosity of the fluid, according to the Hagen-Poiseuille equation:
\[ Q = \frac{\Delta P \times \pi r^4}{8L} \times \frac{1}{\eta} \]

where \( Q \) is the volume flow rate; \( P \) is the pressure gradient; \( r \) and \( L \) are the tube radius and length respectively; and \( \eta \) is the coefficient of viscosity of the fluid.

The geometric resistance of the tube is \( 8L/\pi r^4 \). Resistance is the reciprocal of conductance, and viscosity is the reciprocal of fluidity.

Using the Hagen-Poiseuille equation as a first approximation for blood flow in an organ,

Volume rate of driving pressure (P artery - P vein) blood flow = Vascular resistance \times blood viscosity

Hence organ blood flow depends on the driving blood pressure gradient, and on the flow resistance, which is partly the geometric resistance of the conducting blood vessels (vascular resistance or vascular hindrance), and partly the flow resistance of blood (blood viscosity), which arises from internal friction between molecules and cells in the blood. The factors which determine local blood flow through the eye are perfusion pressure, resistance vessel diameter, and vascular blood viscosity. The capillary and retinal perfusion pressures in normal adult humans is in the range of 35-50mmHg. (Grunwald, 1986) The effect of changes in perfusion pressure on retinal blood flow is damped by autoregulation.
The microvascular geometry in any organ is complex and varies greatly between tissues. It is also dynamic, since arterioles especially can rapidly and markedly alter their diameter, and thereby alter flow distribution: for example, in response to local metabolic requirements (autoregulation) or temperature (thermoregulation). Venous resistance can also be greatly increased, for example by venous thrombosis, or by increased tissue pressure, which tends to collapse the veins.

The flow resistance of the blood (viscosity) is not a simple constant but is complex and dynamic. Blood cells continuously interact with each other and with the plasma proteins. These interactions are influenced not only by the intrinsic composition of the blood (which can be measured in a venous blood sample), but also by the extrinsic physical conditions. Of particular importance are the geometric constraints of the blood vessel (especially its diameter), and the flow rate. These two factors interact to cause shearing, which is relative movement of adjacent layers (streamlines) of flowing blood. The shear stress is the force per unit area causing this relative movement within the blood, and the shear rate is the velocity gradient between adjacent fluid layers. The mean shear rate in a vessel is calculated thus,

\[
\text{Mean shear rate} = \frac{4 \times \text{velocity (m/sec)}}{\text{radius (in)}}
\]

Hence, the viscosity of blood in a narrow, rapidly perfused vessel differs greatly from the viscosity of the same blood in a wide, slowly perfused vessel. Vessel diameter not only influences the shear forces within the blood, but also requires
deformation of individual red and white blood cells when it decreases below the cell
diameter (6 to 8 μm), as in retinal capillaries: this also greatly alters the flow
resistance of the blood.

4.1.2 Blood Rheology

Rheology (Greek, rhein, to flow) is the study of flow and deformation of
matter. Blood rheology (haemorheology) deals with the flow and deformation of
blood and blood vessels. Bloods prime function appears to be transport by flow
(irreversible deformation), and its most important macrorheological property is its
resistance to flow, or viscosity.

The flow behaviour of individual blood cells (microrheology) is more relevant
to the understanding of retinal blood flow. This includes cell deformation,
aggregation between cells, and adhesion of cells to the vessel wall. The flow
behaviour of blood in large-bore vessels (bulk blood viscosity) is very different from
its flow behaviour in microvessels (i.e., capillary tubes or micropore filters whose
pore diameter is of the same order as red or white blood cell diameter).

Most clinical studies of blood rheology have concentrated on two global tests
of the flow behaviour of whole blood: blood viscosity, measured in wide-bore
viscometers; and blood filtrability through micropore filters. Each of these global
tests measures in part blood flow properties which can be measured more specifically
(plasma viscosity, red cell aggregation, and red or white blood cell deformation), and
in part changes in blood composition, e.g., haematocrit (the volume fraction of cells in
blood), which can also be directly measured.
4.1.3 Blood viscosity

Viscosity of a liquid is its resistance to flow, due to internal friction. In a streamline flow of a simple liquid in a cylindrical rigid tube, theoretical concentric cylindrical layers of fluid slide over each other, undergoing shearing.

Resistance to shearing arises from friction between adjacent fluid layers’ and the fluid's viscosity is this flow resistance. The shear stress ($\tau$) is the force per unit area applied to a fluid layer which produces movement relative an adjacent layer. The shear rate ($\gamma$) is the velocity gradient between adjacent layers. Isaac Newton proposed that viscosity of a fluid was constant regardless of the flow conditions. Blood, however, varies its viscosity with shear conditions, and is termed a 'non-Newtonian fluid’. In a non-Newtonian fluid the viscosity coefficient is calculated as the overall shear stress to the overall shear rate, and is termed correctly the ‘apparent viscosity’.

Determinants of Viscosity

Whole blood viscosity is a global measure that is dependent on cellular and plasma factors, such as plasma viscosity, haematocrit, temperature, and shear rate: the shear dependence of blood viscosity is a function of red cell aggregation at low shear rates and red cell deformation at high shear rates. Whole blood viscosity has little use in routine clinical diagnosis, but it does have some predictive value for blood flow behaviour in large vessels in vivo. When corrected for the effects of haematocrit and plasma viscosity, blood viscosity at high and low shear rates can be used as measures
or red cell aggregation and deformation. However, a cheaper and more practical alternative is to measure red cell aggregation directly, with devices such as the Myrenne Aggregometer. A reduction in blood viscosity by isovolumetric haemodilution is known to increase retinal blood flow. (Kohner et al. 1979)

Individual determinants of blood viscosity are described below.

4.1.3.1 Plasma Viscosity

Since whole blood is a suspension of cells in plasma, its viscosity is a function of plasma viscosity. At any haematocrit, increases in plasma viscosity are associated with increases in blood viscosity. (Rand et al. 1970; Mayer, 1966)

Plasma proteins influence high-shear blood viscosity by their effect on plasma viscosity, (Rand et al. 1970; Mayer, 1966) and further increase low-shear blood viscosity by their effect on red cell aggregation. The important effect of fibrinogen on plasma viscosity probably explains reports of correlations of fibrinogen level with high-shear blood viscosity, reports of increase in high-shear blood viscosity on addition of fibrinogen, and reports of decreased high-shear blood viscosity following therapeutic reduction in fibrinogen levels. In a study by Engstrom and co-workers plasma viscosity was found to be raised in HIV-infected individuals. (Engstrom et al. 1990)

4.1.3.2 Haematocrit

Progressive addition of red cells to plasma progressively disturbs its flow streamlines, and hence blood viscosity increases with haematocrit (volume fraction of
cells, or packed cell volume [PCV]). When studied over a haematocrit range of 0.20 to 0.60, which includes the great majority of human values in health and disease, there is a logarithmic increase in blood viscosity with a linear increase in haematocrit. (Chien et al. 1966; Weaver et al. 1969; Dormandy, 1970) At haematocrits greater than 0.60, this relationship does not appear to hold. Within the normal range of haematocrit (0.35 to 0.55), the relationship to blood viscosity might equally well be linear. (Chien et al. 1966)

These variations in haematocrit effect on viscosity with shear rate can be explained by Chien's concept of effective cell volume. (Chein, 1972) At low shear rates, an increase in red cell concentration promotes aggregation of red cells, which increases effective cell volume, disturbs flow streamlines, and hence blood viscosity. At high shear rates, an increase in red cell concentration promotes mutual deformation of red cells, which decreases effective cell volume and hence partly compensates for the increase in viscosity.

4.1.3.3 Temperature

Blood viscosity is usually measured as a predictor of blood flow in vivo: hence it is usually determined at the physiological temperature of 37°C. Additional measurements at lower temperatures have been made in Raynaud's syndrome, (Goyle and Dormandy, 1976) and in cryoglobulinemia, (Harkness, 1971) (Harkness and Philips, 1981) in both of which abnormal patterns of viscosity-temperature response have been observed. Plasma viscosity increases by 2.4% with every degree Celsius fall in temperature below 37°C.
4.1.3.4 Shear Rate

Blood is a non-Newtonian fluid, i.e., its viscosity varies with shear rate. At high shear rates, normal blood viscosity falls (shear-thinning) to reach a minimum value of about 4 to 5 mPa sees, i.e., 3 to 4 times higher than plasma viscosity. The microrheological effect for this shear-dependence of blood viscosity is twofold: red cell deformation at high shear rates and red cell aggregation at low shear rates.

4.1.3.5 Red Cell Deformation

Red cells are not rigid particles, but fluid droplets with low internal viscosity. The importance of red cell deformation in reducing viscosity at high shear rates was shown by viscometric studies of poorly deformable sickle cells. Such hardened red cells exhibit a high viscosity without shear-dependence, because they cannot deform and neither can they aggregate, since deformation of the red cell membrane is required for aggregation.

In the rat retinal model it has been found that reduced retinal perfusion of red blood cells occurred in some areas of retinal pathology, which may be due to the RBC not being able to pass through narrowed vessels (which would be related to RBC deformability), (Ben-Nun et al. 1990) or it may be due to leukocyte plugging which was not commented on in the paper.

4.1.3.6 Red Cell Aggregation

Fahraeus (Fahraeus, 1929) observed that normal red cells in plasma form linear aggregates (rouleaux) under low flow conditions. Red cell aggregation results from the action of large plasma proteins, especially fibrinogen, which form bridges
between adjacent red cells and overcome their mutual repulsion due to negative charges on their surfaces. Primary rouleaux undergo secondary aggregation (end-to-end, or end-to-side) to form networks. These red cell aggregates disturb the flow streamlines in plasma, hence red cell aggregation by fibrinogen is largely responsible for the increase in blood viscosity at low shear rates. (Chien et al. 1966; Weaver et al. 1969; Fahraeus, 1958) Viscosity still increases to some extent at low shear rates without fibrinogen, due first to loss of red cell deformation, and second to aggregation of red cells by serum globulins such as α-2-macroglobulin and immunoglobulins. Zeta sedimentation ratios (a measure of red cell aggregation) has been found to be raised in HIV-infected individuals. (Engstrom et al. 1990) The severity of conjunctival disease in HIV-infected individuals has been found to be associated with increased zeta sedimentation ratios and fibrinogen levels. (Engstrom et al. 1990)

4.1.4 Blood Viscosity in Narrow Vessels

While blood viscosity, measured in wide-bore viscometers, is a global index of blood flow resistance in large vessels, it underestimates blood flow resistance in narrow vessels such as retinal capillaries, which impose geometrical constraints on individual blood cells. This is shown by the much greater flow resistance of blood (or blood cell suspensions) when filtered through sieves with narrow pores, which mimic the geometrical constraints of the microcirculation.

Studies of the microcirculation in vivo have confirmed the considerable deformation of both red and white cells in capillaries. In a system used in clinical studies, whole blood samples or diluted cell suspensions are filtered through
polycarbonate or metal filters, with straight pores of 3 to 8 μm diameter and approximately 10-20 μm length. Such methods model sections of capillaries which red or white cells have been observed to plug in vivo, i.e., capillary entrances, branch points, or stenoses (e.g., where an endothelial cell nucleus protrudes into the capillary lumen). These techniques have also been used to demonstrate decreased red or white cell deformability in disease states such as sickle cell anaemia; renal disease, liver disease, and hyperlipoproteinaemia; hypertension; and diabetes. These results may be relevant to the shortened red cell survival or the occlusive microvascular complications of these diseases. Studies on whole blood viscosity in HIV-infected individuals have not been performed to date.

These filtration systems have also shown the considerable ability of white cells to block capillaries. White cells have much higher viscosity than red cells, due largely to their rigid nuclei. Whole blood filtration, using filters of 5 μm pore diameter, measures pore plugging by white cells and red cells in equal measure, since the former are 700 times less frequent, but 700 times more likely to plug pores. The clinical significance of white cell deformability in the nutritive microcirculation is illustrated by the leukostatic complications of hyperleukocytic leukaemias, the incidence of which correlates with the reological properties of leukaemic cell types. White blood cells have found to be less deformable in patients with NIDDM than a normal control group. (Pecsvarady et al. 1994) Studies have therefore been undertaken to look at the role of pentoxiphylline in the treatment of diabetic microvasculopathy as this drug can improve retinal blood flow by affecting WBC deformability amongst other actions. (Sonkin et al. 1993a; Sonkin et al. 1993b; Armstrong et al. 1990; Sonkin et al. 1992)
Blood filtration is a global test of blood's potential flow resistance in models of the microcirculation. Like blood viscosity, it is influenced by temperature, shear forces, haematocrit, plasma viscosity, red cell deformability, and red cell aggregation. The filter geometry, driving pressure, cell concentration, and time of measurement vary greatly in different measurement systems, and hence can give widely differing results. The development of a simple technique for whole blood filtration through polycarbonate filters (pore diameter approximately 5 μm) at high driving pressures (-20 cm water)(Reid et al. 1976) led to a large number of clinical studies in various disease states. While many associations with disease, prognosis, and treatment were observed, the global nature of the test makes it difficult to determine the relative contributions of red cells, white cells, platelet aggregates, and plasma proteins.(Lowe, 1981) Filtration studies of relatively pure populations of red cells or white cells are therefore required for assessment of their individual contributions to capillary flow resistance.

4.1.5 White Blood Cell Rheology

Leukocytes are larger and more rigid than erythrocytes (Moessmer and Meiselman, 1990) and have been shown to have a strong influence on microvascular blood flow(Sutton and Schmid-Schonbein, 1992). In addition, polymorphonuclear leukocytes (PMN), which comprise the largest fraction of leukocytes, are capable of causing direct microvascular damage by the release of proteases and toxic oxygen radicals (Weiss, 1989). Leukocytes are probably the main reservoir for latent CMV, (Lang et al. 1977; Cassol et al. 1989; Stanier et al. 1989) altered rheological
properties of leukocytes may therefore contribute to the risk for CMV retinitis. Little is known about the rheological behaviour of leukocytes from such subjects.

The diameter of WBC in suspension is comparable to the major diameter of the discoid RBC, which is 7 to 8 μm. (Schmid-Schonbein et al. 1984) The diameters of the WBC as seen on blood smears, however, are usually larger than 10 μm. This is due to the flattening of the sphere into a thin pancake shape by the stresses applied during smear preparation. Although the diameter of the WBC in suspension is about the same as the major diameter of the RBC, the spherical shape of WBC leads to a larger cell volume than that of RBC.

The effects of chemical modifications of the cytoskeletal apparatus of the WBC on their viscoelastic properties have been studied by using colchicine and cytochalasin B. Colchicine is an agent that can cause disruption of the microtubules. In comparison to the control, colchicine causes a dose-dependent increase in deformation in response to the same step aspiration pressure. Experiments have also been performed with the use of cytochalasin B, which disrupts the actin microfilaments and colchicine which can disrupt microtubules. These experiments suggest that both the microfilaments and microtubules are important in governing the passive viscoelastic properties of the WBC; their disruption leads to a decrease in the viscoelastic coefficients of the cell.
4.1.6 Rheological properties of neutrophils in their active state

Neutrophils obtained from fresh heparinized blood samples and suspended in a Ringer-albumin solution tend to deform spontaneously without an external deforming force. They develop membrane projections (protopods) which lead to irregular cell shapes. (Schmid-Schonbein et al. 1981)

Biochemical analysis of extracts of horse leukocytes, guinea pig granulocytes, (Stossel and Pollard, 1973) and rabbit macrophages (Hartwig and Stossel, 1975) indicates that actin-like and myosin-like proteins are present, as well as other proteins which interact to control their polymerisation. Gelation of these proteins appears to generate the formation of the protopod and the change in the mechanical properties of the cell. The neutrophils from HIV-infected patients have been found to have enhanced activation, increased actin polymerization and increased expression of adhesion molecules CD11b/CD18. (Elbim et al. 1994)

Leukocyte flow behaviour in retinal or narrow vasculature

In order to understand the dynamics of the retinal circulation, the study of the way WBC behave in narrow vessels is particularly relevant. When rheological tests on WBC are made with large deformation involving the whole cell (Lichtman, 1973; Miller and Myers, 1977), the geometry of the cell and the deformation of the nucleus may become significant factors in determining the viscoelastic behavior of the WBC. Calculations on the basis of surface area and cell volume show that the WBC have more excess surface area than RBC and that the WBC can pass through cylindrical pores as small as 2.6 to 2.8 μm, i.e., about the same limiting value for RBC passage. However, this is based on steady state behaviour; in order to attain this type of
deformation, the time factor involved between WBC and RBC is quite different because of their differences in cellular viscosity and hence the time constant of dynamic deformation.

Leukocytes, however, are often found to roll along the vessel wall, adhere to it, or even actively move in the opposite direction to the flow along the wall, if there is local damage or the presence of chemotactic agents. The WBC are especially prone to come into contact with the endothelium in the venular system, as a result of several haemodynamic mechanisms. As RBC leave the capillaries and enter venules in converging Y streams, they tend to overtake WBC, and in the process force the WBC towards the vessel wall. (Schmid-Schönbein et al. 1980) Similarly, a WBC leaving a capillary and entering a venule at a T-junction would be swept by the mainstream in the venule in such a way that it tends to be pressed against the venular wall downstream of the capillary. When the flow rate is slow and RBC aggregation occurs, the WBC are displaced toward the vessel wall by the rouleaux, and the WBC concentration is reduced in the central region. This is another mechanism by which WBC can be marginated. As a result of these haemodynamic mechanisms and WBC-RBC interactions, the WBC can come into close contact with the endothelial wall, leading to the possibility of forming temporary bonds. There they may adhere or roll on the wall at a lower velocity than the main blood stream. This interaction of WBC with the vessel wall can be an important mechanism of abnormally high resistance to blood flow in inflammation, shock, infection and other low flow state.
4.1.7 Flow of leukocytes through vessel branches

There are considerable spatial and temporal nonhomogeneities of blood cell distribution the microcirculation. The flow velocity in capillary networks also exhibits significant spatial and temporal fluctuations (Wayland and Johnson, 1967). Krogh (Krogh, 1922) observed that the RBC concentration in a daughter vessel of a divergent branch varies with the rate of blood flow in the vessel and that the haematocrit in small side branches is lower than that in the larger vessel (plasma skimming). In symmetrical model bifurcations with a tube to-particle diameter ratio only slightly above 1, the branch with the faster flow receives proportionally much more particles than expected from the flow ratio (Chien et al. 1985).

On the arteriolar side of the capillary network, the entrance of a WBC into a capillary, by raising the local resistance, causes the preferential entry of subsequently arriving RBC into the other capillary branches. Hence, the presence of WBC modulates the temporal and spatial apportionment of RBC in the capillary network, thus influencing oxygen delivery to tissue cells.

In capillaries with diameter of about 5 μm, each WBC is equivalent to approximately 700 RBC in its contribution to flow resistance. This rheological influence of the WBC in narrow vessels is attributable more to its high viscosity coefficient (about 700-1,000 x that of RBC content) than its elastic property (only about 4 x that of RBC), and probably reflects the time needed for the deformation of the larger and more viscous WBC at capillary entrances. Rheological events at in low flow states favour WBC-endothelial interactions, and WBC adhesion may occur in response to chemotactic factors in the microenvironment. WBC adhesion to
endothelium, by reducing the vessel lumen and flow rate, can lead to further increases in flow resistance, especially with the entry of additional WBC.

The postcapillary venules, which have the lowest shear stress in the circulation, (Chien, 1976) and are formed by converging capillaries, are therefore the site with the rheological and geometric conditions favouring WBC movement toward the vessel wall. Experimentally it is found that some 95% of the WBC in the postcapillary venule roll along the venular wall. (Schmid-Schonbein et al. 1980) This provides the WBC with a strategic location from which they can react to any chemotactic signal from the extravascular space. Furthermore, the slow translational velocity in rolling along the venular wall facilitates the extravasation of the WBC when needed, potentially allowing with it the spread of CMV infection in the case of CMV infected leukocytes.

4.1.8 Adhesion molecules

Several important leukocyte functions are critically dependent on cell-cell interaction, including phagocytosis and chemotaxis, adhesion to platelets and endothelium.

There are a number of groups of adhesion molecules. The selectins are membrane glycoproteins that initiate leukocyte adhesion to the blood vessel wall. The binding of endothelial selectins is relatively weak, and they induce a rolling phenomenon whereby the cells in the blood vessels slow down and roll over the endothelial surface. Selectins are important in the initial interactions of leukocytes
with the capillary walls, but firmer binding and penetration though the capillaries need leukocyte integrins and the ICAMs.

An additional factor that may contribute to microvascular disease in HIV-infected individuals is damage resulting from neutrophil dysfunction. Neutrophils comprise the largest proportion of circulating leukocytes. When activated, they become extremely rigid, (Vermes et al. 1987) and tend to adhere to endothelial cells. Activated neutrophils, trapped in capillaries, may degranulate leading to local tissue damage caused by the release of potent proteases and O2 radicals. (Weiss, 1989; Lang et al. 1977; Sandborg and Smolen, 1988) A recent study evaluated the following parameters in HIV-infected patients: (1) Expression of cellular adhesion molecules, L-selectin and the β2 integrin CD Iib/CD18, which are involved in adherence to endothelial cells and transendothelial migration to inflammatory sites; and (2) actin polymerization (Elbim et al. 1994), which is postulated to mediate receptor mobilisation and the oxidative burst. Unstimulated neutrophils from HIV-infected individuals were shown to express increased levels of CD 11 b/CD 18 adhesion molecule, decreased levels of L-selectin antigen expression, and increased actin polymerization. (Elbim et al. 1994) In addition, impairments in L-selectin shedding were associated with decreased CD4+ T-lymphocyte counts. (Elbim et al. 1994) Excessive or inappropriate stimulation of neutrophils may contribute to vascular damage. Infection of endothelial cells with a clinical isolate of CMV has been found to induce enhanced production of the neutrophil chemoattractant C-X-C chemokines, interleukin-8 and GROalpha. Infected endothelial cell supernatants have been found to induce neutrophil chemotaxis in a transendothelial migration assay. Neutrophils can acquire infectious CMV during transmigration across infected endothelium and
are subsequently able to transmit infectious virus to fibroblasts. CMV-infected endothelial cells can recruit neutrophils by the secretion of C-X-C chemokines and can transmit the virus to them by direct cell-to-cell contact and during neutrophil transendothelial migration, suggesting that the neutrophil-endothelial cell interaction plays an important role in virus dissemination in vivo.(Grundy et al. 1998)

Haemodynamics and cellular-endothelial adhesion are interdependent. Initial capture of a white cell onto endothelium requires the molecular binding with ICAM-1/CD18 as well as sufficient contact duration. Further bond formation between white cell and endothelium is dependent on local shear stress and tensile forces on the bond. Leukocyte-endothelial interactions can be viewed therefore at both a molecular and hydrodynamic level.

4.1.9 Coagulation abnormalities

It has been proposed that low-grade disseminated intravascular coagulopathy in severely immunosuppressed individuals with HIV-infection and secondary infectious, inflammatory, or neoplastic complications is responsible for depressed protein C levels that indicates a thrombotic predisposition.(Feffer et al. 1995)

Human protein S is a 75-kD vitamin K-dependent plasma glycoprotein that acts as a cofactor for activated protein C in the anticoagulant cascade.(Esmond, 1987) Protein S deficiency can occur in HIV infected patients associated with thrombosis. The proinflammatory cytokine, tumour necrosis factor- alpha (TNF-alpha), has been reported to be elevated in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients and has been shown to induce a
procoagulant state on the surface of endothelial cells. In vitro TNF-alpha has been shown to downregulate protein S synthesis and this may be the mechanism for localized procoagulant activity and thrombosis in HIV-infected patients. (Hooper et al. 1994) Cytomegalovirus infection in vitro of both rat and human umbilical vein endothelial cell monolayers have been shown to induce procoagulant activity. (van Dam-Mieras et al. 1992) The importance of this effect in vivo has yet to be established. Antiphospholipid antibodies are commonly found to be elevated in patients with HIV disease, the clinical relevance of this has also to be determined.

Deficiencies of protein C, protein S and anti-thrombin III have all been associated with ocular vascular occlusive disease. (Bertram et al. 1995) A procoagulant state in HIV-infected patients may lead to venous obstruction leading to increased resistance in the ocular vasculature and decreased flow.

4.2 Measureable Rheologic Parameters

4.2.1 Erythrocyte Sedimentation Rate

Erythrocyte sedimentation rate (ESR) was first studied in detail by Farhreus, who showed that the main reason for an increased rate of settling was clumping of the erythrocytes. The ESR test is usually carried out using the Westergren method. For the standardised Westergren method the ESR at a given temperature, depends on the following variable, 1) size of the cell, 2) the difference in density between the blood
cell and the suspending plasma, 3) the plasma viscosity, 4) the extent of aggregation
of blood cell elements.

Stokes equation describes the movement of particles in a fluid

\[ V = \frac{2ga^2(d_1 - d_2)g}{9\eta} \]

where \( a \) = surface area of sphere, \( d_1 - d_2 \) is the differential density of the sphere
and the fluid, \( g \) is force of gravity and \( \eta \) is viscosity.

\( V \) = velocity of equilibrium of a sphere, which is proportional to the mass of
the sphere, therefore if red cells aggregate due to increased plasma protein content
overcoming the normal negative charge (zeta potential), the aggregated clumps of cell
will effectively increase the mass of each individual particle and sediment more
rapidly.

Stoke’s law does not accurately predict clinical ESR values for a number of
reasons. The concentrations of the red cells in blood is such that the hydrodynamic
perturbations of the suspending fluid produced by the suspending blood elements
reduces the rate at which other elements subside. Hence the influence of haematocrit
on ESR. Red cell shape differs also considerably from a sphere and the rate of
suspension is heavily influenced by the mean size of the subsiding units.

4.2.2 Zeta Sedimentation Ratio

Under controlled conditions of centrifugation the mutually repelling negative
charge (zeta potential) on red cells can be overcome.(Bull and Brailsford, 1972)
The most sophisticated of the improvements in sedimentation analysis is the zeta sedimentation rate (ZSR), a measure of the packing of erythrocytes under a standardised stress (zetacrit). The zeta potential results from negatively charged sialic acid groups on the red cell membrane. (Bull and Brailsford, 1972) As noted earlier, the separating effect of the negative charge upon adjacent-red cells is decreased in the presence of fibrinogen.

The forces of alternating compaction and dispersion are produced by the Zetafuge (Coulter Diagnostics, Hialeh, Florida), a centrifugal device. A blood volume of approximately 100μl is placed in a vertical capillary tube and spun at 400 rpm, for 45-second intervals over three minutes, rotating the tube 180 degrees at each interval. The resulting forces cause the red cells to follow a zigzag path down the capillary tube and to become more densely packed. The degree of compaction at three minutes is the zeta sedimentation rate, measured as the red cell haematocrit. The ZSR is expressed in per cent rather than millimetres per hour. It assesses the ease with which the red cells will pack under stress and is presumably related to the zeta potential of these cells when suspended in a particular plasma. It has major advantages over the ESR, as it is unaffected by anaemia and responds in an approximately linear fashion throughout the range of clinical significance. The capillary tubes are easier to fill, require, less sample, and are cheaper than the standard ESR tubes, and the overall testing period lasts less than five minutes.

The tremendous advantage of the ZSR technique is that it greatly increases the extent of any potential aggregation. This process minimises the effect of fluctuations.
in plasma viscosity and diminishes the role of haematocrit in determining subsidence rates.

4.2.3 Plasma Viscosity

Plasma can be viewed as a complex solution of macromolecules and salts. The viscosity of such a system is both a colligative property of the solution and a constitutive property of the macromolecules. (Hess and Cobure, 1950) The measured viscosity of the plasma is a function of many variables, the more important of which are: i) the concentration of the solute (macromolecules), ii) the shape and intramolecular flexibility of the macromolecules, iii) the electric charge borne by the macromolecules, iv) the degree of solvation of the macromolecules, v) the viscosity of the solvent (water), vi) the temperature and pH of the solution, vii) the ionic strength of the solution, viii) the shear rate regime under which the viscosity is measured.

Thus, while the viscosity of plasma is primarily determined by the number of macromolecules present, their intrinsic properties and the properties of the solvent also heavily influence the viscosity of the solution.

The viscosity of plasma depends almost entirely on the concentration of the various macromolecules present and therefore serves as a means of detecting changes in the concentrations of plasma proteins. The sensitivity of the technique, in terms of detecting changes in the concentration of a particular macromolecule, will depend on the nature of the specific macromolecule changes. Changes in the concentration of asymmetric macromolecules such as fibrinogen are more readily detected than
changes in the concentration of smaller more symmetrical macromolecules such as albumin (Hardwick and Squire, 1952).

4.2.4 Immunoglobulins and Immune Complexes

Retinopathy is a feature of the hyperviscosity syndrome. The most common types of paraproteinaemia are Waldenstrom's IgM macroglobulinaemia and multiple myeloma of type IgG and IgA. Visual findings include engorged veins or occluded veins, sludging of red cells in the capillaries, haemorrhages, exudates and microaneurysms have also been reported (Foulds and Ashton, 1962; Fahey et al. 1965; Luxenbourg and Mausolf, 1970) As in polycythaemia, the retinopathy of paraproteinaemia may be due to either increase blood viscosity or to increased blood volume. The retinopathy, as with other features of paraproteinaemia can be reversed by plasma exchange, which lowers both blood viscosity and blood volume (Luxenbourg and Mausolf, 1970)

HIV-infected patients are known to have a polyclonal increase in serum immunoglobulin levels (IgG, IgM, and IgA), and the presence of autoantibodies. The circulating level of immunoglobulins have been shown to be normal (Ammann et al. 1983) or increased (Euler et al. 1985) Different techniques that are used to detect immune complexes use different components of the complexes, which may explain the discrepancies in the published reports. In a study by Engstrom and co-workers mean IgG levels were found to be increased for all grades of conjunctival microvasculopathy, but no statistically significant difference was found. Mean IgA and IgM levels were not found to be increased (Engstrom et al. 1990)
Immune complexes are usually measured using Raji cells, which are B lymphoid cells that express no surface immunoglobulin but bear surface receptors for the complement (C3b, C3d, and possibly C1q) and the Fc portion of Immunoglobulin. Raji cells bind immune complexes that can subsequently be detected by the addition of an labelled anti-immunoglobulin antibody. The Raji-cell assay has been found to be more sensitive than C1q-binding assay in detecting elevated levels of circulating immune complexes in AIDS and AIDS-related complex.(Gupta and Licorish, 1984)

4.2.5 Red cell aggregation

The reversible aggregation of red blood cells, is a primary determinant of the rheologic behaviour of blood;(Stoltz and Donner, 1987; Lowe, 1987) at low shear rates, RBC aggregation is the major cause of the non-Newtonian flow properties of normal blood. In addition, the in vivo flow dynamics of blood are influenced by red cell aggregation,(Chein, 1987) and several reports have indicated abnormal aggregation in such clinical situations as cardiogenic pulmonary oedema, diabetes, myocardial ischaemia, renal failure, thrombo-embolic states and venous thrombosis.(Bauersachs et al. 1987; Hein et al. 1987; Dormandy and Nash, 1987) In many diseases, the concentration of aggregation-promoting plasma proteins is increased, aggregates form more rapidly at stasis, and the shear forces necessary to disperse the aggregates are higher than in healthy controls.(Stoltz and Donner, 1987; Lowe, 1987) The elevated degree of RBC aggregation which can be observed at stasis is often associated with an enhanced ability of low shear rates to promote RBC aggregate formation. Since in many pathophysiological situations subnormal shear
forces may exist in portions of the circulatory system,(Stoltz and Donner, 1987; Chein, 1987) an increased tendency for RBC aggregation is of significant clinical relevance.

It is generally accepted that RBC aggregation in due to bridging between adjacent cells by specific plasma proteins (e.g., fibrinogen, globulins); it can also be induced by large water-soluble macromolecules such as dextrans with a molecular weight greater than about 70,000.(Nash et al. 1987) However, Brookes and co-workers have questioned this bridging hypothesis and have proposed a thermodynamic model based on polymer/protein depletion from the intercellular contact zone.(Brooks and Janzen, 1988) Red blood cell aggregation can be considered as a dynamic process and thus represents a balance between the forces of aggregation and the forces of disaggregation (e.g., mechanical shear, electrostatic repulsion between cells, resistance of the cells to deformation during aggregate formation).

Before the development of an automated and interactive system, based on a Myrenne aggregometer, which allows rapid and routine measurement of several RBC aggregation indices and which requires only a small sample volume, specific indices of RBC aggregation were difficult to determine, as techniques were technically demanding to use.

The methodology used to determine the various RBC aggregation indices follows from the earlier studies of Schmid-Schonbein and co-workers, in that it is based increase of light transmission through a red cell suspension which occurs when individual cells aggregate into rouleaux or rouleaux-rouleaux complexes; gaps in the
suspending medium between aggregates allow more light to pass through the suspension. Increased light transmission also occurs at high shear rates (i.e., in the absence of RBC aggregation) due to formation and-alignment with flow. (Schmid-Schonbein et al. 1972) The resulting transmission-shear rate data thus exhibit a minimum between stasis or low shear rates and high shear rates, with this lowest point indicating the minimum shear rate necessary to disperse pre-existing RBC aggregates. (Bauersachs et al. 1999)

### 4.2.6 Summary of Global Measures of Aggregation

Analysis of extensive data in the literature indicate that changes in plasma viscosity primarily reflect alterations in the concentration of plasma macromolecules. The erythrocyte sedimentation rate (ESR) and zeta sedimentation ratio (ZSR) tests mainly detect the presence of macromolecules in the plasma, which produce rouleaux or aggregates. The ESR test as presently used is, however, more prone to produce data supporting erroneous conclusion than the ZSR. (Seaman et al. 1981) Seaman and co-workers have suggested eliminating ESR measurements and use only plasma viscosity and ZSR measurements in clinical evaluations. (Seaman et al. 1981)

### 4.3 Clinical Studies

Two related studies were undertaken to determine if differences in selected rheologic parameters exist between HIV infected patients with CMV retinitis and those without. The first study explored neutrophil deformability and the second study
looked at various other rheologic measures that primarily affect red cell aggregation. The studies were carried out separately due to availability of equipment, although many patients were entered into both studies.

**Study 1 Neutrophil Deformability**

**Aim**

To see if abnormal neutrophil deformability exists in HIV infected individuals, and whether there is a difference in deformability between patients with and without CMV retinitis.

**4.3.1 Materials and Methods**

A Cell Transit Analyser was used to measure the transit time of individual PMN through 8 μm pores, where an increased transit time indicates decreased PMN deformability. PMN transit times were studied for the following groups: HIV-infected patients (n=47) (without CMV disease n=33, and with CMV retinitis n=14), and HIV-negative patients (n=19). Patients with diabetes mellitus, or blood transfusions within the last month were excluded from the study. Patients were recruited from The Jules Stein Eye Institute, and The CARE Clinic, University of California Los Angeles (UCLA) Medical Center. Volunteers without known HIV-infection served as controls and were tested during the same time period with the same methodology. The Study was approved by the UCLA Human Subjects Protection Committee, and all subjects gave informed consent.
A medical history was obtained and a physical examination was performed on all patients. At the time of blood draw the patient underwent ophthalmic examination and blood pressure was measured. Blood was drawn for the following studies: PMN deformability, leukocyte count, cholesterol, creatinine and triglycerides. Lipids were checked as severe hyperlipidaemia can occur with certain HAART regimes which may alter leukocyte membrane properties and hence deformability. Creatinine was checked as renal disease has been associated with abnormal red cell rigidity. With the exception of PMN deformability, all tests were carried out using standard clinical laboratory methods.

4.3.1.1 PMN Preparation

Whole blood was gently drawn into a syringe containing sodium heparin (10U/ml blood) using a 21 gauge needle, with the tourniquet removed immediately after insertion of the needle. Pure PMN suspensions were prepared using a density-based centrifugal technique; endotoxin-free sterile media and plastic ware were used to avoid in vitro PMN activation. In brief, 7 ml of heparinized whole blood was mixed with 3 ml of phosphate buffered saline (PBS pH = 7.4, 285mOsm/kg), gently layered onto 5 ml of 50% Percoll (Sigma, St. Louis, MO), and then centrifuged at 400g for 25min at 22°C.

The top 3 mls of the resulting PMN and RBC interface and pellet was transferred into distilled water for 30 seconds in order to remove residual erythrocytes by hypertonic lysis. Tonicity was then restored by the addition of 10x sterile PBS (Sigma) and the suspension spun at 220g for 6min at 22 °C. The PMN were washed and maintained at room temperature in PBS. Purity was assessed by optical
microscopy (>95% PMN), and leukocyte count was determined with an automated haematology analyser.

4.3.1.2 PMN deformability measurements

PMN cellular deformability (i.e., the ability of the entire cell to adopt a new shape in response to deforming forces) was assessed using the Cell Transit Analyser (CTA, ABX Hematologie, Montpellier, France). (Pecsvarady et al. 1992; Koutsouris et al. 1989; Fisher et al. 1992) The central feature of the CTA is the special filter that separates two fluid-filled reservoirs. The filter is a specially-fabricated thin sheet of polycarbonate plastic containing 30 identical pores, and thus represents a simple yet geometrically stable in vitro model of a capillary bed.(see Figure 4.1)(Moessmer and Meiselman, 1990; Pecsvarady et al. 1992) Hydrostatic pressure generated by the difference in height between the two fluids forces the cell suspension through the filter pores, and the transit time for each complete cell passage is obtained by monitoring the change in electrical resistance of the filter as a cell traverses a pore. Software for the computer system corrects for possible simultaneous pore occupancy, and provides transit time statistical data (i.e., mean, median) for the cell population being tested. A longer PMN pore transit time reflects a decrease of PMN deformability.(Moessmer and Meiselman, 1990; Pecsvarady et al. 1992)
Schematic representation of CTA. A dilute suspension of PMN in PBS buffer is placed in reservoir A, with PBS in reservoir B; the pressure gradient (8 cm H₂O), resulting from the height difference between the two fluid columns, forces the suspension through the 8μm pores of the filter. The two needle electrodes are connected to an AC conductimeter that measures the resistance of the filter, digitizes the signal and outputs the digital information to a computer for computation of the PMN transit time.
For our CTA studies, the following experimental parameters were employed: 1) 8 micron diameter by 21 micron long pores; 2) pressure gradient of 8cm water; 3) PMN final concentration in the sterile PBS of $10^5$ cells/ml; 4) median transit time of at least 1000 cells determined for each run, with each sample run in triplicate and the average of these three median values taken as the final result. All CTA measurements were carried out at 25 °C using the same 8 by 21 micron filter, and all studies were completed within 4 hours following venipuncture. In order to check for possible temporal changes of the CTA filter characteristics with repeated use, control samples of patients with a known range of PMN transit times were processed with each batch of samples.

4.3.1.3 Statistical Analysis

All data are presented as means +/- standard error (SE). Comparisons between the three groups were performed by analysis of variance (ANOVA). The Tukey-HSD test was used for intergroup comparisons. The relationship between variables was analyzed by Pearson simple correlation.

4.3.2 Results

All patients with CMV retinitis were receiving systemic anti-CMV drugs at the time of examination. No patients had cotton-wool spots or retinal haemorrhages.
PMN from HIV-infected individuals had longer mean transit times (5.56 +/- 0.23 msec) than HIV-negative controls (4.02 +/- 0.16 msec, p<0.001). There were no significant differences between these groups in terms of age or blood pressure (all p values ≥0.47) (see Table 4.1).

Among the HIV-infected patients, PMN from patients with CMV retinitis had longer mean transit times (6.91 +/- 0.33 msec) than HIV-infected patients without CMV retinitis (5.01 +/- 0.24 msec, p<0.001) (see Table 4.2). There were no significant differences between these subgroups in terms of age, systolic blood pressure, or serum creatinine, cholesterol, or triglycerides (all p values ≥0.20). Individuals with CMV retinitis had higher diastolic blood pressure (p=0.02) and lower CD4+ T-lymphocyte counts (p=0.04) than those without CMV retinitis. Mean HIV viral load were higher in patients with CMV retinitis, although the difference was not statistically significant (p=0.26).

The mean transit time of PMN from HIV-infected patients with CD4+ T-lymphocyte counts > 100/gl (4.36 +/- 0.23 msec) was not significantly different from that of controls (4.02 +/- 0.16 msec, p=0.25). There were no significant differences between these two groups in terms of age or blood pressure (all p values ≥0.36).

Among all HIV-infected individuals, transit times were inversely correlated with CD4+ T-lymphocyte counts (r = -0.61, p< 0.001)(see Figure 4.3).

Transit times were not correlated with age, blood pressure, HIV blood level, or serum creatinine, cholesterol, or triglycerides (all p values ≥ 0.09). The relationships of CMV retinitis and CD4+ T-lymphocyte count to pore transit times were considered in a regression analysis; both CD4+ T-lymphocyte count and CMV
retinitis were significant predictors of increased transit time when all patients were considered. Thus, the relationship between CMV retinitis and transit time could not be explained solely on the basis of low CD4+ T-lymphocyte counts.

Although sample sizes are small, it was informative to look at subgroups based on CD4+ T-lymphocyte counts. In five of the 11 patients with CMV retinitis for whom CD4+ T-lymphocyte counts were available,4 counts were >50/µl, the threshold above which CMV retinitis usually does not occur. Values for these five patients were 52, 61, 78, 154, and 300/µl. It is therefore likely that these patients were receiving combination antiretroviral therapy and had experienced elevations in CD4+ T-lymphocyte counts after CMV retinitis developed. PMN from these five individuals had a mean transit time (6.80 +/- 0.48 msec) that was nearly identical to the value for patients with CMV retinitis whose CD4+ T-lymphocyte counts were <50/µl (n=6, 6.76 +/- 0.57 msec, p=0.96). Among HIV-infected individuals whose CD4+ T-lymphocyte counts were <50/µl, the mean transit time for PMN from those without CMV retinitis (n=7, 6.56, +/-0.27 msec) was not significantly different from mean transit time for patients with CMV retinitis (n=6, 6.76 +/- 0.57 msec, p=0.76). The relationship between CD4+ T-lymphocyte count and pore transit time remained significant when only HIV-infected individuals without CMV retinitis were considered (r = -0.63; p=0.0001).

4 Some routine clinical laboratory data such CD4 counts were unavailable as many of the patients were referred by outside physicians, who could not always provide accurate or current data.
Table 4.1 Comparison of HIV-infected individuals and HIV-negative Controls

<table>
<thead>
<tr>
<th></th>
<th>HIV-Positive Individuals (n=45)</th>
<th>HIV-Negative Controls (n=17)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39.1 +/- 1.2</td>
<td>37.5 +/- 2.2</td>
<td>0.47</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
<td>130 +/- 2.1</td>
<td>128 +/- 3.7</td>
<td>0.67</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
<td>76 +/- 1.6</td>
<td>74 +/- 3.8</td>
<td>0.67</td>
</tr>
<tr>
<td>Transit time:++(msec)</td>
<td>5.56 +/- 0.23</td>
<td>4.02 +/- 0.16</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

sBP=systolic blood pressure.
dBP=diastolic blood pressure.

* Two-sample tests allowing for unequal variances.

+ Data are means +/- standard errors.

++ In vitro polymorphonuclear leukocyte transit time in a Cell Transit Analyzer as a measure of leukocyte rigidity.
Table 4.2 Comparison of HIV-Infected Individuals with and without CMV retinitis

<table>
<thead>
<tr>
<th></th>
<th>Individuals With CMV Retinitis (n=13)</th>
<th>Individuals Without CMV Retinitis (n=32)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.1 +/- 2.3</td>
<td>38.2 +/- 1.4</td>
<td>0.26</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>131 +/- 3.3</td>
<td>129 +/- 2.7</td>
<td>0.75</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.7 +/- 1.8</td>
<td>73.5 +/- 2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.27 +/- 0.22</td>
<td>0.93 +/- 0.05</td>
<td>0.20</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>189 +/- 4.0</td>
<td>191 +/- 3.5</td>
<td>0.96</td>
</tr>
<tr>
<td>Serum triglycerides (mg/dl)</td>
<td>392 +/- 226</td>
<td>468 +/- 68.5</td>
<td>0.04</td>
</tr>
<tr>
<td>CD4+ T-lymphocyte count</td>
<td>68.4 +/- 26.5</td>
<td>175 +/- 27.3</td>
<td>0.04</td>
</tr>
<tr>
<td>HIV blood level</td>
<td>217,000</td>
<td>50,400</td>
<td>0.26</td>
</tr>
<tr>
<td>(genomes per ml)</td>
<td>+/- 126,000</td>
<td>+/- 25,600</td>
<td></td>
</tr>
<tr>
<td>Transit time: (msec)</td>
<td>6.91 +/- 0.33</td>
<td>5.01 +/- 0.24</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

sBP=systolic blood pressure. DBP=diastolic blood pressure.

*Two-sample tests allowing for unequal variances.
+Data are means +/- standard errors.
++In vitro polymorphonuclear leukocyte transit time in a Cell Transit Analyzer as a measure of leukocyte rigidity.
Figure 4.3 Correlation between transit time and CD4+ T-lymphocyte count

Polymorphonuclear leukocyte rigidity (expressed as in vitro transit times in a Cell Transit Analyser) versus CD4+ T-lymphocyte count in HIV-infected individuals. There is a statistically significant correlation ($r = -0.61$, $p < 0.0001$). Individuals without CMV retinitis are indicated with solid dots; individuals with CMV retinitis are indicated with open dots.
4.3.3 Discussion

Studies of the rheologic behaviour of blood and its formed elements may provide insight into the pathogenesis of HIV-related diseases of the retina. When PMN are activated, f-actin content increases, the cells become more rigid, and they can obstruct flow in capillaries. (Sutton and Schmid-Schonbein, 1992) Activated PMN also tend to adhere to endothelial cells and may degranulate, leading to local vascular endothelial damage mediated through the release of potent proteases and \( O_2 \) radicals. Thus, rigid PMN might damage microvasculature with permanent alterations of blood flow.

Abnormal PMN rigidity has been found to occur in patients with diabetes mellitus,(Pecsvarady et al. 1994) who have microvascular changes similar to those seen in patients with AIDS, and in a feline model of diabetes mellitus.(Braun et al. 1996) The increased PMN transit times found in our study indicate that PMN are also more rigid in HIV-infected individuals. Although factors such as age, blood pressure, and serum creatinine, cholesterol, and triglyceride might affect PMN rigidity, the relationship identified in this study could not be explained on the basis of those factors.

The mechanism of decreased PMN deformability in HIV-infected individuals is unknown. It might be due to changes in membrane lipid composition, (Masuda et al. 1990) or due to a state of chronic PMN activation, caused by HIV infection or other stimuli. PMN from HIV-infected individuals have been shown to express increased levels of CD11b/CD18 adhesion molecule, decreased levels of L-selectin
antigen expression, and increased actin polymerisation, all of which suggest chronic PMN activation. (Elbim et al. 1994)

Use of filgrastim (granulocyte colony stimulating factor) to elevate PMN counts is common among HIV-infected patients receiving ganciclovir or other drugs that cause bone marrow suppression. Subjects were not questioned about the use of filgrastim or other leukocyte growth factors, and therefore any effect of such agents on PMN rigidity is unknown.

PMN rigidity was related to CD4+ T-lymphocyte count, but no association was found with HIV viral loads, another indicator of the severity of HIV-related immunosuppression. The relationship between CMV retinitis and PMN rigidity was not solely a reflection of the fact that CMV retinitis occurs at low CD4+ T-lymphocyte counts. This observation should be interpreted with caution, however, because CD4+ T-lymphocyte counts could have changed during the course of the retinal infection, attributable to initiation of potent antiretroviral therapy. This study was performed in 1996 before many patients were receiving protease inhibitors, and we did not control for use of antiretroviral therapy, which increases CD4+ T-lymphocyte counts. We did observe, however, that counts were above 50/ml (the threshold above which CMV retinitis usually does not develop) in five of 11 patients with CMV retinitis; from these data, we can infer that their counts had risen after development of CMV retinitis, in response to antiretroviral therapy. Transit times remained high in the subgroup of patients with CMV retinitis and high CD4+ T-lymphocyte counts. If one hypothesised that CD4+ T-lymphocyte counts were the sole predictor of PMN rigidity, then elevation of counts should return transit times to lower values, and one would expect to see transit times that are lower in patients with
CMV retinitis and counts >50/μl than in those patients with CMV retinitis whose counts remain <50/μl. We did not see evidence of this pattern, however. The fact that transit times were nearly identical for these two subgroups of patients suggests that once PMN rigidity is increased, it may not return to normal, even with improved immune function, placing HIV infected patients at continued risk for retinal vascular disease. The half-life of a PMN in the circulation is measured in hours, indicating that there must be a state of chronic PMN activation in the subpopulation of patients with CMV retinitis.

Haematogenous spread of CMV to retinal tissue is supported by the findings that CMV retinitis frequently develops adjacent to retinal blood vessels, and the fact that development of CMV retinitis in both patients with AIDS and other immunosuppressed patients is related to CMV viraemia. It is interesting to speculate that haemorheologic abnormalities might facilitate the development of CMV retinitis, by slowing the transit of infected leukocytes through the retinal vasculature or by increasing the interaction between CMV-infected leukocytes and vascular endothelium. Although a relationship between CMV retinitis and transit time that is separate from the effect of low CD4+ T-lymphocyte counts was not found when evaluating only patients with counts <50/μl sample sizes were small, and our study was not designed to investigate a causal relationship between PMN rigidity and CMV retinitis. Our population was not necessarily representative of patients at the onset of CMV retinitis; CD4+ T-lymphocyte counts and transit times may have changed subsequent to disease onset. Conversely, CMV disease might be a cause, rather than the effect, of PMN activation and increased rigidity. The activation state of PMN in patients with AIDS and CMV retinitis are currently unknown, but PMN have been
identified as the site of early replication of CMV during reactivation from latency. (Gerna et al. 1992)

Even in the absence of intraocular opportunistic infections or other clinically apparent retinal lesions, HIV-infected patients can have visual abnormalities, including visual field defects, changes in colour vision, and altered contrast sensitivity. It is possible that these subtle changes in vision might be attributable to haemorheologic abnormalities and the retinal microvasculopathy of HIV infection. In diabetics, loss of contrast sensitivity has been related to retinal capillary dropout. (Arend et al. 1997) With the increased survival of HIV-infected individuals attributed to potent antiretroviral therapies, it is increasingly important to understand the pathogenesis of HIV-related retinal microvasculopathy and its associated clinical abnormalities.

In summary, leukocyte rheology offers a novel approach to the study of retinal microvascular disease and its clinical sequelae in HIV-infected individuals. Our data has identified an association between the mechanical behaviour of PMN and chronic CMV retinitis that should be clarified in future studies. Also, leukocyte rheology may provide a link to understanding the similarity between the retinal microvasculopathy of AIDS and diabetic retinopathy.
Study 2 - Other Rheologic Measures

Hypothesis

Abnormal blood viscosity is associated with the development of CMV retinitis.

Aim

To see if measurable peripheral blood factors known to affect viscosity (haematocrit level, zeta sedimentation ratio, fibrinogen level, plasma viscosity, circulating immune complexes, and quantitative immunoglobulins) are associated with the presence of CMV retinitis in HIV-infected individuals.

4.4.1 Materials and Methods

The following groups were studied: HIV-infected patients (n=42) (without CMV disease n=31, and with CMV retinitis n=11), and HIV-negative patients (n=17). Patients with diabetes mellitus, or blood transfusions within the last month were excluded from the study. Patients were recruited from The Jules Stein Eye Institute, and The CARE Clinic, University of California Los Angeles (UCLA) Medical Center. Volunteers without known HIV-infection served as controls and were tested during the same time period with the same methodology. The UCLA Human Subjects Protection Committee approved the Study, and all subjects gave informed consent.

A medical history was obtained and a physical examination was performed on all patients. At the time of blood draw the patient underwent ophthalmic examination
and blood pressure was measured. Blood was drawn for the following studies: leukocyte count, cholesterol, creatinine and triglycerides. With the exception of, all tests were carried out using standard clinical laboratory methods.

4.4.1.1 Laboratory Studies

For each patient the following values were obtained: haematocrit level, zeta sedimentation ratio, fibrinogen level, plasma viscosity, circulating immune complexes, and quantitative immunoglobulins.

Venous blood from the anticubital vein was drawn through a 20-gauge needle into a 40-ml syringe without anticoagulant, after the release of the tourniquet, for more than five seconds to minimise the stasis effects on viscosity. For test to be performed on plasma, blood was immediately transferred into the appropriate anticoagulant: ethylenediaminetetraacetate for haematocrit and zeta sedimentation ratio; sodium citrate for fibrinogen level; and sodium heparin for plasma viscosity. Tests for circulating immune complex levels and quantitative immunoglobulin testing were performed on the serum.

Haematocrit levels were determined immediately after drawing blood, using a zetafuge and microhematocrit centrifuge. Red cell aggregation and zeta sedimentation ratio was performed on blood adjusted to a haematocrit of 0.40 to adjust for any possible haematocrit-induced error. (Deng et al. 1994) Red cell aggregation was determined by using a Myrenne Aggregometer System. Circulating immune complexes were determined by Raji cell assay, plasma viscosity, and fibrinogen levels by the Clauss Method.
4.4.1.2 Myrenne Aggregometer System

In the present study light-transmission data for various conditions were measured using a transparent cone-plate instrument (Model MA-1. Myrenne GmbH, Roetgen, FRG); the one has a nominal two degree angle, the plate is standard 25 by 75 mm glass microscope slide, and an infra-red light emitting diode-photo detector system allows measurement of light transmission through the suspension contained in the gap between the cone and plate.

4.4.1.3 Statistical Analysis

All data are presented as means +/- standard error (SE). Comparisons between the groups was performed by analysis of variance (ANOVA). Assumptions made in testing were checked for, including equality of variances. The relationship between variables was analysed by Pearson simple correlation.

4.4.2 Results

Clinical characteristics of the patient groups are shown in Table 4.3 There were no significant differences in any of the patient characteristics measured except for CD4+ T-lymphocyte count. HIV positive patients with CMV disease tended to have a higher serum creatinine and HIV load and a lower CD4+ T-lymphocyte count than HIV positive patients without CMV disease. A distinction between active and inactive CMV retinitis was not made in this study. All patients with CMV retinitis were receiving specific anti-CMV therapy at the time of examination.
Table 4.3 - Subject Groups

<table>
<thead>
<tr>
<th></th>
<th>HIV+,CMV-</th>
<th>HIV+,CMV+</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Individuals studied</td>
<td>31</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>38.8 +/-7.3</td>
<td>40.7 +/-8.7</td>
<td>NS</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
<td>130 +/-14</td>
<td>130 +/-11</td>
<td>NS</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
<td>75 +/-11</td>
<td>80 +/-4</td>
<td>0.44</td>
</tr>
<tr>
<td>leukocyte count</td>
<td>4.7 +/-2.2</td>
<td>3.9 +/-2.2</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Creatinine (mg/dl)</td>
<td>0.93 +/-0.15</td>
<td>1.27 +/-0.22</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Cholesterol (mg/dl)</td>
<td>200 +/-71</td>
<td>184 +/-90</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Triglycerides (mg/dl)</td>
<td>468 +/-237</td>
<td>398 +/-220</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ count (cell/µl)</td>
<td>178 +/-29</td>
<td>75 +/-32</td>
<td>0.03</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>41.5 +/-4.8</td>
<td>34.4 +/-8.8</td>
<td>0.002</td>
</tr>
<tr>
<td>IgA</td>
<td>420 +/-284</td>
<td>776 +/-700</td>
<td>NS</td>
</tr>
<tr>
<td>IgG</td>
<td>1702 +/-377</td>
<td>1793 +/-531</td>
<td>NS</td>
</tr>
<tr>
<td>IgM</td>
<td>108 +/-41</td>
<td>115 +/-75</td>
<td>NS</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>13.6 +/-2.2</td>
<td>11.5 +/-2</td>
<td>NS</td>
</tr>
<tr>
<td>Red cell aggregation</td>
<td>13.0 +/-3.8</td>
<td>14.1 +/-4.8</td>
<td>NS</td>
</tr>
<tr>
<td>ZSR</td>
<td>0.65 +/-0.08</td>
<td>0.68 +/-0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (md/dl)</td>
<td>322.1 +/-86.8</td>
<td>283 +/-109.0</td>
<td>NS</td>
</tr>
<tr>
<td>Viscosity</td>
<td>1.87 +/-0.19</td>
<td>1.97 +/-0.14</td>
<td>NS</td>
</tr>
<tr>
<td>HIV load (genomes/ml)</td>
<td>50400 +/-25600</td>
<td>103200 +/-69800</td>
<td>NS</td>
</tr>
</tbody>
</table>

sBP = systolic blood pressure, dBP = diastolic blood pressure

NS means non-significant, P>0.05

Data are means +/- SD
The mean values for HIV infected patients with CMV retinitis was greater than both mean values for HIV infected patients without CMV retinitis and the upper limit of the normal range for ZSR (0.68 : 0.65 : normal, 0.46-0.58), IgA (776:420: normal, 75-326 mg/dl), IgG (1793:1702, normal, 650-1449mg/dl).

The mean values for HIV infected patients with CMV retinitis was greater than mean values for HIV infected patients without CMV retinitis but within the normal range for plasma viscosity (1.97:1.87: normal, 1.5-1.9), IgM (115:108: normal, 28-207mg/dl).

The mean values for HIV infected patients with CMV retinitis was less than mean values for HIV infected patients without CMV retinitis but within the normal range for fibrinogen (283:322: normal 200-400mg/dl). The mean haematocrit for HIV infected patients with CMV retinitis (34%) was significantly less (p=0.002) than HIV-infected patients without CMV retinitis (42%).

Both zeta sedimentation ration and red cell aggregation were inversely correlated with CD4+ T-lymphocyte count (P=0.15 and P=0.006 respectively).

4.4.3 Discussion

Haematogenous spread of CMV to retinal tissue is supported by the findings that CMV retinitis frequently develops adjacent to retinal blood vessels,(Holland and Shuler, 1992) and CMV-like particles have been found within macrophages in the choroidal circulation in a patient with AIDS and CMV viraemia. In addition,
development of CMV retinitis in other immunosuppressed patients is related to CMV viraemia. (Fiala et al. 1977)

Studies of the rheologic behaviour of blood and its formed elements may provide insight into the pathogenesis of HIV-related microvasculopathy and CMV retinitis. Red blood cell aggregation is the primary factor causing increased blood viscosity at low flow rates, with fibrinogen being the plasma protein most responsible for red cell aggregation. (Repolge et al. 1967) Using the conjunctiva as a model, the severity of microvascular disease in patients with HIV infection has been shown to be associated with increased red blood cell aggregation and fibrinogen levels. (Engstrom et al. 1990) The presence of cotton-wool spots in patients was also associated with elevated fibrinogen levels. (Engstrom et al. 1990) These haemorheologic abnormalities in patients with HIV infection may contribute to decreased blood flow that may facilitate CMV infection by increasing contact time between circulating infected cells and retinal tissue and by breakdown of the blood retinal barrier. (Engstrom et al. 1990). The presence of cotton wool spots has been associated with the subsequent development of CMV retinitis. (Jabs, 1995)

Rheologic abnormalities may contribute to both vascular endothelial damage (Engstrom et al. 1990) and sludging of blood which may modulate the CMV load in retinal vessels. The combination of an effective localized increase in CMV load in retinal vessels, due to decreased flow, and microvascular endothelial damage may increase the risk of the development of CMV retinitis.

These cellular changes might be involved in the pathogenesis of HIV-related microvasculopathy and CMV retinitis.
Chapter 5 Retinal Capillary Blood Flow

5.1 Aim of studies looking at retinal capillary blood flow

Hypothesis

We hypothesise that abnormal retinal capillary blood flow exist in HIV-infected individuals and that blood flow is relatively more impaired in patients who have or are about to develop CMV retinitis. Impaired blood flow may be due to abnormal blood constituents and/or due to vascular damage.

Aim

To see if an association exists between altered retinal capillary blood flow measured in vivo, and the presence or absence of CMV retinitis in HIV-infected individuals. If impaired blood flow is present, this finding may support the notion that sluggish retinal capillary blood flow is a risk factor for developing CMV retinitis, by increasing leukocyte-endothelial contact time. Although such studies are not designed to show a causal relationship, if associations are found then further longitudinal studies may be performed to see if such measures may become a clinically useful non-invasive marker in predicting disease and in the understanding of pathogenesis.
5.2 Introduction

5.2.1 Retinal Blood Flow

The mammalian retina has been found to possess an unusually high rate of glycolysis (Winkler, 1981) and oxygen consumption (Kimble et al. 1980; Weiter and Zuckerman, 1980), with this activity fuelling the active transport processes that maintain the ionic gradients necessary for visual transduction and electrical activity. The continuous oxygen and metabolic substrate demand of the retina require a high rate of blood flow.

5.2.1.1 Regulation of Retinal Blood Flow

Vascular Supply of the Retina

The retinal vessels are distributed in the inner part of the inner retina, extending outward from the optic disc in all directions. The retinal vessels enter the eye through the optic disc and branch radially into the retinal tissue primarily within the nerve fibre layer along the inner limiting membrane.

The retinal arterioles do not have sphincters on the arteriolar bifurcations; Muller cells and astrocytes extend over large areas in close spatial relationship with retinal vessels. The retinal arterioles give rise to capillary intercommunicating networks, one lying within the nerve fiber and ganglion cell layer, a deeper one within the inner nuclear layer, and finally a superficial capillary network distributed around the optic disc and along the temporal superior and inferior retinal vessels. (Henkind et al. 1973) There is no autonomic innervation of the retinal and optic nerve head.
vessels, although α- and β-adrenergic and cholinergic receptors (Alm, 1993) and receptors for angiotensin are present. (Ferrari-Dileo et al. 1987)

Autoregulation of Retinal Blood Flow

The retinal blood flow is autoregulated by modifications of the vascular resistance, following changes of the contractile state of the retinal arterioles. The mechanism underlying the autoregulation of retinal blood flow is a balanced result of a metabolic and a myogenic component.

By means of a myogenic mechanism, the retinal blood flow is maintained constant despite moderate variations in perfusion pressure. The ocular perfusion pressure (mean arterial pressure-intraocular pressure) can be modified by either modifications of the systemic arterial pressure or of the intraocular pressure. With the rise or fall of the perfusion pressure beyond normal levels, the arterioles normally constrict or dilate, respectively, to regulate the blood flow to constant values; however, autoregulation becomes ineffective when the perfusion pressure rises or falls beyond certain limits. Using laser Doppler velocimetry and monochromatic fundus photography, it was shown that the retinal blood flow is autoregulated up to a mean systemic blood pressure increase of 41% above baseline values (Robinson et al. 1986) or during an increase in intraocular pressure (above 27-30 mm Hg) leading to a mean retinal perfusion pressure decrease of less than 50%. (Riva et al. 1986) The stimulus for a myogenic mechanism is a variation in the transmural pressure difference, during moderate variations in perfusion pressure, and it is achieved by changing the vascular resistance. (Alm, 1993)
The concept of a metabolic control of the retinal blood flow is that factors on which retinal metabolism is dependent (retinal-tissue PO$_2$, (Riva et al. 1983) PCO$_2$, (Tsacopoulos and David, 1973) pH, nitrous oxide, (Hudes et al. 1988), prostaglandins, and endothelin (Rolinski et al. 1994; Geier et al. 1995)) strive to optimise retinal blood flow according to the metabolic needs of the retinal tissue.

5.2.1.2 Retinal blood flow in HIV-infected patients

Acquired immunodeficiency syndrome microvasculopathy occurs in the absence of cytomegalovirus retinitis and is not accounted for by immunosuppression alone. (Glasgow and Weisberger, 1994) The location and character of these vascular changes in AIDS indicate an ischaemic pathogenesis. In AIDS patients with cytomegalovirus retinitis, the vascular changes are more marked than patients without disease, and include capillary destruction. (Glasgow and Weisberger, 1994)

Techniques that allow for in vivo measurement of blood flow in the retinal microcirculation would therefore be most relevant clinically.

5.2.2 Techniques of measuring retinal blood flow in humans

Many established techniques that measure retinal blood flow are not designed to measure flow at the capillary level. These techniques include the use of: 1) radiolabeled microspheres that measures tissue perfusion rate and is the “gold standard” for measuring retinal blood flow, but suffers from not being able to be used in humans, and from the use of microspheres that are larger that capillaries, 2) laser Doppler velocimetry, an objective reproducible technique, that measures the Doppler
shift of light scattered by erythrocytes, but cannot, however, measure velocity in small vessel and requires assumptions about the velocity profile in the vessels, 3) the measurement of mean circulation time, (the transit time of blood through the retinal circulation) by taking densiometric measurements of serial photographs, which is a technique requires the use of many assumptions including those of capillary volume. 4) Magnetic Resonance Imaging (MRI) is a non-invasive imaging technique used clinically to examine soft tissue. Berkowitz and co-workers are currently working to use a high field research MRI to map ocular oxygenation. (Berkowitz, 1997)

The three techniques that can measure capillary blood flow are described in more detail below.

5.2.2.1 Blue Field Entoptic Simulation

The blue field entoptic phenomenon refers to the effect created by gazing into a brightly illuminated blue field of light (wavelength of 430 nm). that allows the viewer to visualise the movement of leukocytes through the perimacular capillaries. (Sinclair et al. 1989)

---

5 Because the laser light is reflected by moving objects (blood cells), its frequency is shifted according to the Doppler effect. The shift in frequency is proportional to the velocity of the moving blood cells. The distribution of the velocities of blood cells can be seen in the Doppler shifted frequency spectra. The final down field spike in the frequency spectra marks the greatest observed blood cell velocity. The spectra then falls to the level of background noise. From this final frequency, originally identified by the operator but more recently by an automated computer algorithm, the maximum velocity, or Vmax, reached by blood cells can be calculated. This, in turn, has been used to calculate the average blood cell velocity, operator} but more recently by an automated computer algorithm, the maximum velocity, or Vmax, reached by blood cells can be calculated. This, in turn, has been used to calculate the average blood cell velocity,
In 1980, Riva and Petrig introduced their Blue Field Entoptic Simulator, which permitted the quantification of perimacular leukocyte velocity and density. (Riva and Petrig, 1980) The instrument allows subjects to view their entoptic image and then attempt to match the motion of simulated leukocytes on a computer screen to that of their own leukocytes.

While blue field entoptic simulation is an obviously subjective test, repeated measures in the same subjects have been shown to be reproducible. (Yap and Brown, 1994) Nevertheless, an investigator must rely on the subjects' ability to reliably perform a psychophysical matching task that may depend on the subjects' cognitive skills. It is unclear to what extent subjects are capable of recreating what they actually see entoptically by adjusting the simulation. Furthermore, the relationship between the recreated perceived leukocyte velocity and density, and the actual speed and prevalence of leukocytes in the perimacular capillaries is unknown. This and the lack of an actual blood flow measure from the technique make its quantitative results difficult to interpret.

Because of the entirely non-invasive nature of the psychophysical task involved and the simplicity of use, blue field entoptic simulation has found many applications, for example, leukocyte velocity has been found to be decreased in patients with diabetic retinopathy. (Rimmer et al. 1989)

5.2.2.2 Scanning Laser Angiography

The introduction of the scanning laser ophthalmoscope in the early 1980s brought quantitative angiography to new level. (Mainster et al. 1982; Webb and Hughes, 1981) This instrument overcomes many of the limitations of traditional
photographic or video angiography. The incandescent light source has been replaced with a thin, low power, scanning argon laser beam that offers the advantages of better penetration through lens and corneal opacities. The resulting images are similar to those obtained with standard video angiography, but with improved spatial resolution and contrast. As such, they can be analysed to obtain haemodynamic measurements such as arteriovenous passage time and mean dye velocity by observing changes in, dye intensity on successive video frames.(Mainster et al. 1982; Webb and Hughes, 1981)

While scanning laser ophthalmoscopy clearly represents major improvement in retinal imaging and angiography, the instrument is expensive and requires a sophisticated user with skill and experience to obtain high-quality angiograms. In addition the need for intravenous injection of dye, means that this procedure may make it unacceptable to many patients.

5.2.2.3 Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) was introduced by Riva et al in 1989.6 The principles of Laser Doppler Flowmetry have been described in detail in the literature.(Bonner and Nossal, 1990) LDF is an extension of laser Doppler velocimetry (LDV) technology. The Doppler effect is the frequency shift of waves emitted or reflected by moving objects. The frequency shift is proportional to the

velocity of the moving object. However, the velocity of the moving red blood cells is extremely small as compared to the speed of light and the resulting Doppler shift can only be measured indirectly by analysing the interference pattern produced by reflected light that has been frequency shifted by hitting a moving red cell and light reflected from stationary objects, e.g. vessel wall. The spectra of the returning light contains frequencies which have been Doppler shifted from the illuminating frequency. Each spike in the frequency spectra of reflected light represents a velocity. In LDF the volume of these spikes indicates the number of particles moving at that velocity. By integrating the frequency spectra, the number of particles moving at each velocity is known. This yields a direct measurement of flow.

There are two drawbacks to LDF the lack of specificity in volume examined, and variable penetration depths of the laser. The user is able to position the laser over any volume of interest, but any visible or hidden vasculature within that volume will contribute to the flow measurement. Two volumes of tissue equally perfused with oxygen will give drastically different flow values if one contains a hidden arteriole and one does not.

Translucent tissue will allow deep penetration of the laser light. This means that the sample volume will be large. Since a large sample volume will contain more capillaries than a smaller volume, the reflected light will contain more Doppler shifted signal and report high flow. Less translucent tissue will not allow the laser light to penetrate as deep, resulting in a smaller sample volume and so a reported lower flow. Use of confocal optics (as in Scanning Laser Flowmetry) may help to reduce the variability by limiting the sample volume optically, but these optics typically only limit the volume to 1/3 mm depth; far larger than the cross sectional height and width.
of the sample volume. The tissue penetration depth is still speculative and under investigation. (See Appendix 8.1 for details of Laser Doppler Flowmetry)

Scanning Laser Doppler Flowmetry (SLDF) (Michelson et al. 1995; Michelson et al. 1995) uses the ability of a laser scanning tomograph (Heidelberg Engineering, Germany) to measure the amount of backscattered light at different locations in the tissue of interest in a very short time. Because it is sufficient for describing a fluctuating signal to take samples from the signal with a sampling rate of at least twice the highest signal frequency, numerous points of the tissue can be examined very quickly by a scanning method. For a single picture line the procedure is as follows: one sample from each point of the line is taken. After a flyback time this measurement is repeated several times for the same points with a high repetition rate. Thus, the backscattered intensities for each scanned point are obtained as a function of time (intensity-time curve). A spectrum analysis from the data of each location is performed using a Fast Fourier Transform (FFT) algorithm. Thus, the power spectrum of the Doppler shift of each retinal point is calculated, by which the blood flow parameters $\omega$ and $\gamma$ can be computed. Performing this sampling procedure point by point yields the map of the blood perfusion of a distinct retinal area. The spatial resolution accuracy of the scanning laser system is 10 $\mu$m. (Zinser, 1993) The examined retinal area has a size of 2.7 x 0.7 mm and is mapped by a resolution of 256 points x 64 lines. (Michelson et al. 1996) A diode laser with a wavelength of 670 nm with an optical powers of 100 $\mu$W was used as the light source. This system examines the retina in a 10° section without pupil dilation using a confocal optical system. Each line is scanned 128 times with a line repetition rate of 4,000 Hz. This leads to an intensity matrix of 256 points x 128 times x 64 lines.
total data acquisition time is 2.048 seconds. (Michelson et al. 1996) Performing an
FFT over the 128 intensity values of each retinal point yields the spectrum of the
Doppler shift of any retinal point. Doppler frequencies of <2,000 Hz can be detected
correctly using a line sampling frequency of 4,000 Hz. Frequencies of <125 Hz were
excluded to minimise the influence of low-frequency artefacts such is breathing,
cardiac action, and intensity fluctuations due to other effects.

The brightness of each pixel is coded by the flow, volume, or velocity value in
the corresponding retinal area (see Figure 5.1).

Roughly speaking the brighter the pixel the faster the cells at this retinal point
move. For quantification of capillary retinal blood flow in terms of Laser Doppler
Flowmetry in relative units the mean value and the histogram of flow, volume, and
velocity may be calculated in any retinal areas of 10 x 10 pixels (100 x 100 µm) in
arbitrary units of the perfusion picture.

In any region of interest all perfusion variables were available (average power
spectrum, histogram of flow, volume, and velocity, mean (standard deviation) of flow,
volume, and velocity, P(f=0) [representing the reflectivity (=DC value)]. The size of
the region of interest is variable (chosen in most studies to be 10 pixels x 10 pixels).
The operator identifies the region of interest in the perfusion picture by 'mouse'.
Using the algorithm the perfusion variables were normalised regarding the reflectivity
by division through P(f=0). Despite this, the DC value of the region of interest
should be in the range of 80 and 150 arbitrary units. Reliable measurements of retinal
perfusion variables were only available in region of interest without larger vessels.
Riva et al showed that the Doppler broadening of capillary vessels in the optic nerve
head and in the retina is maximally 300 Hz. The technique presented can measure Doppler broadening up to 2000 Hz. As the blood velocity of larger vessel, leads to Doppler broadening greater than 2000 Hz, this technique is not able to measure blood flow in larger vessels. Only in regions of interest with Doppler broadenings lower than 2000 Hz are reliable measurements available.

5.2.3 Choice of Technique for this Study

For practical purposes a non-invasive technique was required in view of the multiple studies the HIV infected population has to undergo, this excluded the use of scanning laser angiography. Scanning Laser Doppler Flowmetry for its ease of use and quick acquisition time was the chosen method. Although the blue light entoptoscope has the advantage of measuring leukocyte movement, it excludes a substantial minority of participants (up to 20% of normal test subjects) who are not able to perform the psychophysical task, of perceiving and matching leukocyte movement, required to produce accurate results. The blue light entoptoscope was not therefore used in this initial study.
Figure 5.1 SLDF example output colour coded by the volume, flow and velocity

The white box encompasses the sampled area of interest, that is selected by a cursor. A flow measurement is then generated for the sampled area.

Volume=15.521
Flow=251.514
Velocity=0.916
5.3 Scanning Laser Doppler Flowmetry

Validity of method

In experimental setup in vitro Michelson and co-workers, (Michelson et al. 1996) were able to show that the SLDF was able to quantitatively measure velocity in absolute units. In their experimental set-up, the velocity of a moving plane with different velocities was examined. The observed and expected frequency shift for the different velocities showed a significant correlation. The SLDF measures decreased retinal and optic nerve head blood flow when increasing the negative pressure inside a suction cup to increase the intraocular pressure in 3 human subjects. (Michelson and Schmauss, 1995) Measurements of flow using the SLDF have also been validated on capillary tubes and animal models (personal communication with Dr Blwantray Chauhan, Dalhousie University, Halifax, Canada).

Michelson and associates have also shown that the SLDF shows a significant and linear relationship to the laser Doppler flowmeter (Oculix), a device that has been previously validated. (Davies et al. 1992; Brein and Riva, 1982) However van Heuven and associates noted that although there was a linear association of RBC velocities between the SLDF and a laser Doppler flowmeter (Perimed) there were discrepancies in volume measured and they noted marked site-to-site variation in the large zero offset. (van Heuven et al. 1996)
5.4 Reliability of method

In a study by Michelson and co-workers (Michelson et al. 1996) in which 5 separate perfusion measurements were performed in 10 eyes of 10 healthy people on 5 consecutive days. The reliability coefficient of flow, volume and velocity were 0.82, 0.81, 0.83 respectively.

5.5 Repeatability and Validity in HIV-infected and non-infected individuals

Aim

Although repeatability studies have been performed on normal and glaucomatous individuals, no repeatability studies have looked at HIV-infected patients. In addition the effect of varying focus, and of cell concentration on flow measurements have not been evaluated. An understanding of the variability of measurements is important when trying to assess differences in flow measurements between groups of patients.

5.5.1 Repeatability in HIV-infected individuals

Nine separate perfusion measurements were performed in 6 eyes of 6 healthy persons and in six eyes of six HIV-positive patients, with no clinical signs of retinal infections (CD4+ T-lymphocyte count 31-486, mean 184). The blood flow was measured at the same retinal locations of each eye at each visit at 5 separate sites. The normal controls were all non-smokers, normotensive, and had no history of ocular disease. The right eye was used in all patients. The SDLF was employed to acquire 6 consecutive images at the same location one disc diameter nasal to the optic disc.
disc, a perifoveal measurement and in zones just superior and inferior to the temporal arcade vasculature. A 20° by 2.5° scan field and a 10 by 10 pixel measurement window was used for data acquisition. The same location was identified at each visit by the use of a transparent overlay grid, on which retinal vasculature marking were traced, which allowed for correct grid alignment. No visible retinal vessels were present in the sample area.

Statistical analysis was performed using SPSS for windows version 5.1. The Kolmogorov-Smirnov test was used to check normal distribution. The intraindividual variability was characterised by the coefficient of variation (CVAR) as:

\[ CVAR = \frac{\text{standard deviation}}{\text{mean}} \times 100\% \]

5.5.7 Results

The coefficient of variation ranged between 9 and 27.3, mean variation in HIV-negative test subjects was 16.28, and 18.25 in HIV-positive subjects. The coefficients of variations for each patient at a given location are give in Tables 5.1a and 5.1b.
Table 5.1a: The coefficients of variation for each of the non-HIV-infected subjects

<table>
<thead>
<tr>
<th>Region studied</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>macula</td>
<td>9</td>
<td>11.7</td>
<td>21.2</td>
<td>16</td>
<td>16.2</td>
<td>22</td>
</tr>
<tr>
<td>peripapillary</td>
<td>16.3</td>
<td>12.6</td>
<td>13.6</td>
<td>13.3</td>
<td>21.6</td>
<td>25.5</td>
</tr>
<tr>
<td>Superior zone 2</td>
<td>19.6</td>
<td>19.3</td>
<td>16.2</td>
<td>15.5</td>
<td>23.5</td>
<td>12.6</td>
</tr>
<tr>
<td>Inferior zone 2</td>
<td>14.5</td>
<td>13.5</td>
<td>19.6</td>
<td>24.2</td>
<td>22.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Temporal zone 2</td>
<td>21.3</td>
<td>23.6</td>
<td>24.3</td>
<td>16.2</td>
<td>18.5</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Table 5.1b: The coefficients of variation for each of the HIV-infected subjects

<table>
<thead>
<tr>
<th>Region studied</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>macula</td>
<td>12.5</td>
<td>15.6</td>
<td>13.7</td>
<td>15.4</td>
<td>21.2</td>
<td>21.3</td>
</tr>
<tr>
<td>peripapillary</td>
<td>16.5</td>
<td>13.6</td>
<td>14.6</td>
<td>12.3</td>
<td>19.4</td>
<td>14.6</td>
</tr>
<tr>
<td>Superior zone 2</td>
<td>14.6</td>
<td>19.8</td>
<td>12.5</td>
<td>16.4</td>
<td>13.5</td>
<td>13.6</td>
</tr>
<tr>
<td>Inferior zone 2</td>
<td>21.9</td>
<td>8.8</td>
<td>27.3</td>
<td>12.7</td>
<td>13.2</td>
<td>12.9</td>
</tr>
<tr>
<td>Temporal zone 2</td>
<td>24.1</td>
<td>12.3</td>
<td>16.4</td>
<td>24.3</td>
<td>16.2</td>
<td>21.6</td>
</tr>
</tbody>
</table>
5.5.1.2 Discussion

This variation may be due to physiologic fluctuations in regional capillary blood flow with time, or it may be due to variability in the measurement technique, or the apparatus per se. There was no clear trend to greater variability in a particular region, and the amount of variability in a given region did not vary significantly between HIV positive and negative individuals.

5.5.2 Validity

Although many of the capabilities of the SLDF have been evaluated, the effect of defocus, and of cell concentration have not. The capability of this technique to quantitatively measure velocities in absolute units was estimated. In an experimental set-up, the velocity of a plane moving with different velocities was examined by SLDF.

Briefly the apparatus consists of a mounted slide driver linked to a micromanipulator control box. A schematic eye was mounted in front of the target to allow for focusing at the correct plane. The mounted slide was moved at right angles with the incident laser beam with velocities of 0, 0.3, 0.66, and 1 mm/sec. For each velocity, the moving plane was examined by SLDF and volume, flow and velocity in relative units readings were recorded. A 20° by 2.5° field was used in acquiring images and a 50 by 50 pixel area was used in analysis. The mounted plane consisted either of a glass slide, or of slides with smears of blood with the following haematocrit, 0, 10.9%, 21.5 and 43% and repeated measurements were made at both 0
and 1mm/sec. The ratio of cell density on the slides between the three haematocrit specimens was confirmed cell counting using a grid overlay and a microscope. The blood concentration was checked in a micro-haematocrit tube following centrifugation for 4 minutes.

5.5.2.1 Results

The flow measurement was found to be significantly correlated to target speed (p=0.11), but no linear association was found between flow and alteration in focus or alteration in haematocrit of the target (see Figures 5.2a, 5.2b, and 5.2c), note that each plotted point is the median value of 10 separate measurements at each pair of values). There was a flow value measure that was generated even for a moving target and this value was greater if the haematocrit was not zero. However there was little difference found between the flow values when the haematocrit was altered. A flow value was generated even for non-moving target and these values were greater for targets with red cells. This ‘zero-threshold’ value could be the result of Brownian motion of particles on the slide as the LDF makes a non-directional measure of flow and this may explain the higher zero-threshold value with stationary slides with a blood smear that would contain more particles. The large zero threshold may make cross sectional studies less reliable unless this factor could be taken into account.
Effect of altering target speed on measured flow

![Graph](image1)

Figures 5.2a above, and 5.2b below

Effect of altering focus on measured flow

![Graph](image2)

181
Effect of altering haematocrit on measured flow (target at 1mm/sec)
5.6 Main Study: Measurement of Retinal Capillary Flow in HIV Infected Individuals With and Without Cytomegalovirus Retinitis

5.6.1 Aim

To compare retinal capillary blood flow in HIV-infected patients with and without CMV disease. We investigated whether retinal capillary blood flow in HIV-infected patients is abnormal. It has been postulated that abnormal retinal blood flow is a risk factor for the development of AIDS-related CMV retinitis.

5.6.2 Methods

The following groups were studied: HIV+ with CMV retinitis (n=10), CMV+ without CMV retinitis (n=9), CMV with extraocular CMV disease (n=3), HIV-negative controls (n=10). Patients who had diabetes, uncontrolled hypertension, or recent blood transfusion were excluded. Retinal capillary blood flow was measured by scanning Laser Doppler Flowmetry (SLDF, Heidelberg Engineering).

In case of opacities of the cornea, lens, or vitreous the reflectivity as well as the perfusion image showed no details. During the measurement time of 2 seconds the patient had to fixate with the fellow eye a small point in a distance of 2 metres.

All patients were refracted subjectively to determine their spherical error. Patients were then adjusted on the Heidelberg slitlamp and the importance of fixation with the non-measured eye at the given target was explained as well as being
instructed not to following the scanning light with the measured eye. The retinal image was focused on the computer monitor- in each change of position and sensitivity adjusted down to just remove "white noise" areas, then focus was adjusted to achieve the brightest image, and finally the sensitivity dial was readjusted to remove any new "white noise". The patient was then asked to fixate in a number of positions and measurements were taken. Measurements were checked to be free of horizontal streaks indicative of ocular movement before saving the image for subsequent analysis. The following areas were imaged the optic nerve (times 3), macula (times 3), and superior, left, right and inferior mid-peripheral ‘zone 2’ retina.

Ten measurements away from any visible retinal vasculature were taken in the following 20° fields with a 10-pixel area of analysis: peripapillary, perifoveal, and in zone 2 ('peripheral retina') of each quadrant. A transparent grid was overlaid on the perfusion map in order to aid selection of sampling areas without overlap. The lowest and highest values from each set were eliminated from analysis. Only right eyes were used for analysis, unless the patient had CMV retinitis, in which case the uninvolved eye (n=7), or the eye with least disease (n=2) was used for analysis.

Blood was also taken for the measurement of the following factors that may be associated with abnormal blood flow CD4+ T-lymphocyte count, β-2 microglobulin, cholesterol, triglycerides, HIV-load, red cell aggregation, haematocrit and viscosity. Patients were also underwent ophthalmic examination, including checking intraocular pressure by Goldmann tonometry and checking of blood pressure.
5.6.2.1 Statistical methods

Data was entered into a spreadsheet program (Excel) and then imported into a statistical package for analysis. (SAS for windows). The data was analysed together with the support of the statistical department at UCLA Medical Center. The data was explored by initially using descriptive statistics and plotting boxplots of variables to see if a relationship existed between flow and the various measurable blood parameters. Rank-sum tests were performed to see if differences existed between different regions of blood flow measurement and between the two groups of patients.

5.6.3 Results:

Descriptive statistics of baseline comparisons between the groups are displayed in Table 5.2 and flow by region is given in Table 5.3, and are also presented in the form of boxplots (Figure 5.3).
### TABLE 5.2 Descriptive statistics of baseline comparisons between the groups

**HIV positive**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>35.7</td>
<td>3.3</td>
<td>26</td>
<td>46</td>
</tr>
<tr>
<td>choleste</td>
<td>194.6</td>
<td>48.7</td>
<td>142</td>
<td>264</td>
</tr>
<tr>
<td>triglyce</td>
<td>536.2</td>
<td>180.9</td>
<td>265</td>
<td>631</td>
</tr>
<tr>
<td>bp-syst</td>
<td>133.2</td>
<td>10.7</td>
<td>116</td>
<td>154</td>
</tr>
<tr>
<td>bp-dias</td>
<td>81.4</td>
<td>9.3</td>
<td>68</td>
<td>102</td>
</tr>
<tr>
<td>mean-hem</td>
<td>39.0</td>
<td>5.6</td>
<td>32</td>
<td>50</td>
</tr>
</tbody>
</table>

**HIV positive CMV negative**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>34.6</td>
<td>3.1</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td>choleste</td>
<td>200.2</td>
<td>50.1</td>
<td>142</td>
<td>264</td>
</tr>
<tr>
<td>triglyce</td>
<td>509</td>
<td>211.3</td>
<td>265</td>
<td>631</td>
</tr>
<tr>
<td>bp-syst</td>
<td>135.1</td>
<td>10.0</td>
<td>118</td>
<td>154</td>
</tr>
<tr>
<td>bp-dias</td>
<td>80.8</td>
<td>11.8</td>
<td>68</td>
<td>102</td>
</tr>
<tr>
<td>mean-hem</td>
<td>42.1</td>
<td>5.9</td>
<td>33.5</td>
<td>50</td>
</tr>
</tbody>
</table>

**HIV positive CMV positive**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>37.1</td>
<td>3.3</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td>choleste</td>
<td>185.3</td>
<td>55.7</td>
<td>145</td>
<td>249</td>
</tr>
<tr>
<td>bp-syst</td>
<td>131.5</td>
<td>11.7</td>
<td>116</td>
<td>150</td>
</tr>
<tr>
<td>bp-dias</td>
<td>82</td>
<td>7.1</td>
<td>72</td>
<td>98</td>
</tr>
<tr>
<td>mean-hem</td>
<td>35.7</td>
<td>2.7</td>
<td>32</td>
<td>40.5</td>
</tr>
</tbody>
</table>
Table 5.2 Descriptive statistics of baseline comparisons between the groups

continued

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>36.7</td>
<td>5.5</td>
<td>29</td>
<td>49</td>
</tr>
<tr>
<td>bp-syst</td>
<td>132.7</td>
<td>11.9</td>
<td>118</td>
<td>143</td>
</tr>
<tr>
<td>bp-dias</td>
<td>70.7</td>
<td>15.2</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>mean-hem</td>
<td>44.3</td>
<td>3.2</td>
<td>41</td>
<td>5</td>
</tr>
</tbody>
</table>
### Table 5.3 Capillary blood flow by region

<table>
<thead>
<tr>
<th>HIV</th>
<th>R1</th>
<th>R4</th>
<th>R7</th>
<th>R8</th>
<th>R9</th>
<th>R10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POSITIVE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median Flow</td>
<td>225.1</td>
<td>207.1</td>
<td>245.5</td>
<td>262.7</td>
<td>285.7</td>
<td>288.2</td>
</tr>
<tr>
<td>Median Volume</td>
<td>12.6</td>
<td>11.4</td>
<td>12.7</td>
<td>15.6</td>
<td>12.9</td>
<td>13.9</td>
</tr>
<tr>
<td>Median Velocity</td>
<td>0.82</td>
<td>0.76</td>
<td>0.90</td>
<td>1.12</td>
<td>1.05</td>
<td>1.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIV</th>
<th>R1</th>
<th>R4</th>
<th>R7</th>
<th>R8</th>
<th>R9</th>
<th>R10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NEGATIVE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median Flow</td>
<td>224.5</td>
<td>230.9</td>
<td>344.1</td>
<td>283.9</td>
<td>291.6</td>
<td>356.9</td>
</tr>
<tr>
<td>Median Volume</td>
<td>14.1</td>
<td>12.9</td>
<td>17.9</td>
<td>15.7</td>
<td>14.3</td>
<td>19.3</td>
</tr>
<tr>
<td>Median Velocity</td>
<td>0.81</td>
<td>0.84</td>
<td>1.24</td>
<td>1.01</td>
<td>1.05</td>
<td>1.26</td>
</tr>
</tbody>
</table>
Figure 5.3

Boxplots showing the distribution of all flow individual measurements in patients with HIV-infection (1 = region 1, HIV negative patient) and without (1.1 = region 1, HIV positive patient). Regions where the measurements were made are represented as follows, 1 = macula, 4 = peripapillary, 7 = superior zone 2 retina, 8 = temporal zone 2 retina, 9 = inferior zone 2 retina, and 10 = nasal zone 2 retina. For each boxplot, the central line represents the median value of flow in that region and patient type, the box covers the inter-quartile range; and the lines encompass the 2.5-97.5 percentiles. Outlying values (values more than 1.5 box-lengths form the 25th or 75th percentile) are represented by circles.
Capillary blood flow, as measured by LDF, was significantly lower in the macular region in both HIV infected and non-infected individuals relative to other areas sampled. The foveal avascular zone was not a targeted measured area, however, we cannot exclude the possibility that some measurements may have included an enlarged capillary avascular zone. No previous topographical map of capillary flow exists to compare these measurements against. One would expect that high metabolic demand at the macula would be associated with a high capillary blood flow relative to more peripheral regions of the retina.

No statistically significant difference was found in flow measurements between the HIV positive and negative groups as well as between the HIV positive, CMV negative and HIV positive, CMV positive groups. This result may be reflect a true lack of difference in retinal blood flow between the groups or the study design may not have been able to detect a difference as the sample size may have been too small, or the error in measurements due to such factors as variability in ‘zero-offset’, focus, effect of ‘hidden’ arterioles in sample volumes, may have masked any difference. Although there was no statistically significant difference between the groups a trend was noted, with the flow in HIV positive patients being less than HIV negative patients, and the flow in HIV positive, CMV positive patients being greater than in the HIV positive, CMV negative group. In a study by Fallon and co-workers (Fallon et al. 1986) on diabetic patients it was noted that capillary flow (as measured by the blue light entoptoscope) was similar between normal controls and diabetic patients without retinopathy, but it was increased in patients with background retinopathy and reduced in patients with pre-proliferative retinopathy. The increase in flow in patients with background diabetic retinopathy relative to normals was
presumed to be related to the higher metabolic rate in patients with diabetes. (Fallon et al. 1986) The metabolic rate in HIV infected patients is raised also, (Kotler, 1989) and this may account for the observation of slightly greater retinal capillary flow. In both HIV and diabetic patients though, there is the counter force of abnormal blood rheology, retinal vascular damage and capillary closure that one would expect to decrease retinal capillary blood flow. It may be a balance between these two opposing effects that may determine the net effect on retinal capillary blood flow in a given clinical state in both HIV and diabetic patients. Patients with pre-proliferative retinopathy have a significantly reduced macula capillary blood flow compared to those patients with background retinopathy. Fallon and co-workers suggested that this reduction was due to widespread generalised capillary closure leading to an increase in overall resistance in the vascular bed, or possibly also due to a breakdown in autoregulation. (Fallon et al. 1986) An analogous situation may be occurring in HIV infected individuals, with balance of various factors (e.g. blood rheologic factors, degree of capillary closure, metabolic rate, autoregulation) interacting to determine retinal capillary flow at a given stage in the HIV disease process. This may lead, as in diabetics, to stages of disease associated with both increased and decreased retinal capillary blood flow. The interplay of these factors may account for the lack of significant differences in capillary blood flow found between the HIV-positive and negative individuals in this study. The finding that the retinal capillary blood flow in CMV positive patients was greater, but not significantly, than in the HIV positive, CMV negative group is unexpected as a greater degree of capillary closure and damage maybe expected in CMV infected patients reducing flow, however the counter forces of an increased metabolic rate during infection (Kotler, 1989) may
account for the observation. Alternatively there is no real difference in flow between the two groups or the difference was too small to be detectable in this study design. Further studies subgrouping HIV infected patients and also investigating autoregulation of retinal capillary blood flow may be more informative in understanding retinal capillary blood flow in HIV infected patients.

Only two of the measured variables were found to be associated with retinal capillary blood flow, beta-2-microglobulin and corrected red cell aggregation. Beta-2-microglobulin is a 99-amino acid polypeptide that is tightly bound to the surface of nucleated cells and in humans it is typically associated with the heavy chains of MHC class 1. Although beta-2-microglobulin is a non-specific marker for HIV infection, elevated beta-2-microglobulin levels are correlated with progression of HIV disease. (Brew et al. 1996; Strathdee et al. 1996; Hofmann et al. 1990) A decreased flow would be expected with more advanced HIV disease as one may expect more rheologic abnormalities and retinal capillary closure although no association was found with CD4+ T-lymphocyte count and HIV load. The lack of association with CD4+ T-lymphocyte count may be related to the use of protease inhibitors in the study population boosting counts. HIV load undergoes short-term fluctuations, either due to therapy or assay variablility, which may account for the lack of measurable association. Corrected red cell aggregation was positively correlated with retinal capillary flow, which is the opposite of the expected result, which is a decrease in flow with increased red cell aggregation.
5.7 Limitations of the Method

Translucent tissue will allow deep penetration of the laser light, which means that the sample volumes will be large. Since a large sample volume will contain more capillaries than a smaller volume, the reflected light will contain more Doppler shifted signal and report high flow. Less translucent tissue will not allow the laser light to penetrate as deep, resulting in a smaller sample volume and a reported lower flow. Use of confocal optics (as featured in the Heidelberg Engineering unit, Heidelberg, Germany) may help to reduce the variability by limiting the sample volume optically, but these optics typically only limit the volume to \(-\frac{1}{3}\) mm depth; far larger than the cross sectional height and width of the sample volume. The tissue penetration depth is still speculative and under investigation.

The total time of measurement is 2 seconds. Therefore patients with bad fixation may show artefacts in the perfusion picture owing to eye movements (microsaccades) during the measurement, giving a horizontal white line in the perfusion picture. Before and after the microsaccade the fixation of the eye is stable, leading to correct values of the perfusion measurement. In these areas of the perfusion picture retinal vessels and capillaries are sharply imaged. Only during the time of the microsaccade are the examined retinal points moving with a high velocity, leading to extremely high flow rates. In this area all pixels are very bright. Thus, only the offline measurement of perfusion variables below and above the white line of the perfusion picture resembles correct perfusion values.

Two volumes of tissue equally perfused with oxygen will give drastically different flow values if one contains a hidden arteriole and one does not. We tried to
account for this by the systematic elimination of outlier values during the data
analysis.

The scattering processes of stationary and moving particles in retinal tissues
can not be described exactly. Thus it is difficult to interpret the quantitative results
for single measured pixels. The application of the Bonner and Nossal theory leads to
impressive maps of the perfusion of the retina and the optic nerve head. The exact
theory of the scattering processes (number of scattering events, scattering angles)
needed to be investigated in more detail. Nevertheless, the described method yields a
two dimensional index for retinal perfusion.

In both the in vitro study and the in vivo study there were a lot of potential
sources of error, form the zero-offset, the variability related to focus, the accurate
placement of the area of analysis away from arterioles, and the variability of repeated
measures, that the use of this method for the detection of differences of retinal
capillary flow between different groups of patients may not be appropriate. Other
techniques of measuring retinal capillary flow, such as blue light entoscopy, might be
tried in future studies.
Chapter 6 Conclusions

In order for pre-emptive therapeutic strategies to become a reality for the prevention either new CMV retinitis or the reactivation of existing lesions we initially need to be able to predict which patient are of greatest risk of these events. In addition with an increasing choice of both local and systemic therapy we need a rational way of selecting which patients could have their CMV retinitis controlled by local therapy alone, with low risk of systemic disease occurring. In order to be able to develop these therapeutic strategies, new ways of selecting those patients at greatest risk of developing CMV disease needs to be developed. With the advent of HAART, measurement the CD4+ T-lymphocyte count has become a less reliable marker for the prediction of those patients at greatest risk of developing CMV retinitis.

In the studies undertaken in this thesis we found that rises in CMV DNA levels in the peripheral blood was associated with either development or reactivation of CMV retinitis or extraocular disease. We also found an association between abnormal PMN rigidity and the presence of CMV retinitis. Abnormal PMN rigidity may be a risk factor for the development of CMV retinitis or a result of CMV disease. It is possible that abnormal PMN rigidity may both effectively increase the local CMV DNA load in the retinal capillaries, as well as damage vascular endothelium, increasing the risk of developing of CMV retinitis. We found no significant difference in retinal blood flow using SLDF between patients with and without CMV retinitis, which is counter to our hypothesis that poor retinal blood flow is a risk factor for the development of CMV retinitis, although this may be artifactual.
FIGURE 6.1. Proposed interaction of virologic and haemorheologic factors in the pathogenesis of CMV retinitis

The diagram below represents a hypothetical model of how various risk factors may interplay resulting in the clinical manifestation of CMV retinitis.

Legend to Figure 6.1

1. Factors affecting peripheral blood load

From the study on CMV DNA load, rising CMV load is a risk factor for the development of CMV retinitis. High virus load does not always seem to predict the development of CMV retinitis, but may be associated with extraocular disease or subclinical infection. The actual virus load in the capillaries may be modified by a number of factors that may increase or decrease the effective virus load in the retinal vessels. Since clinically and histopathologically retinal infection is mono- or pauci-focal in origin it implies that high virus load alone is not probably sufficient to produce retinal infection and that some disruption to the blood retinal barrier may be necessary to initiate disease.
2. Factors affecting capillary flow

Rheologic abnormalities may contribute to both vascular endothelial damage (Engstrom et al. 1990) and sludging of blood which may modulate the CMV load in retinal vessels, that is increase or decrease the CMV load in the capillaries relative to peripheral venous blood. The combination of an effective localised increase in CMV load in retinal vessels, due to decreased flow, and microvascular endothelial damage may increase the risk of the development of CMV retinitis by increasing leukocyte endothelial contact time.

3. Blood retina barrier breakdown

Excessive or inappropriate stimulation of neutrophils may contribute to vascular damage due to enhanced activation and rigidity of circulating PMN, which may release proteases, from HIV-infected individuals (Gabrilovich et al. 1991; Spear et al. 1990) PMN have been identified as a site of early replication of cytomegalovirus during reactivation (Gerna et al. 1992) and this may possibly contribute to their increased rigidity relative to other HIV infected individuals without CMV disease. Alternatively increased PMN rigidity may increase the risk of developing CMV retinitis due to effect on microvascular blood flow and potential to damage the blood retina barrier. Direct endothelial infection by HIV or CMV may also allow for CMV to pass through the blood retina barrier.

4. Replication in retinal tissue

Immunomorphologic studies by Rao and co-workers (Rao et al. 1998) suggest that retinal endothelial cells are the initial targets for the viral retinitis with subsequent spread of the infection to glia and other retinal cells, including RPE.

5. Clinical disease

The clinical appearance of the CMV retinitis may depend on the interaction of immune response to the virus, type and dose of antiviral therapy and viral pathogenicity. For example a vigorous immune response may give rise to associated vitritis, and macula oedema, where as suboptimal dose of drug may give rise to a mild clinical border ‘activity’.
6 Summary of Results and Discussion

The following section summarises the results and interpretation of the individual studies. Following this we discuss the implication of this work on the clinical management of CMV retinitis as well as any potential application in other areas of ophthalmic disease.

6.1. Quantitative CMV DNA load

6.1.1 Validation of the solution hybridization assay

The specificity for solution hybridization assay and cell culture / shell via assay, was 100%. Solution hybridization assay and cell culture / shell via assay correlated in identifying CMV-positive patients but solution hybridization assay detected more CMV positive specimens earlier in the course of infection in individual patients. Solution hybridization assay is more rapid and sensitive than cell culture / shell via assay for detection of CMV in blood, yields objective quantitative results, and may be useful for the early detection of CMV infection and improved patient management but it is not as sensitive as qualitative PCR assay.

6.1.2 Blood CMV DNA levels in patients without CMV disease at baseline

Patients with high CMV peripheral blood load (>5000 genomes/ml) were more likely to subsequently develop CMV retinitis (3 of 4 patients), or CMV disease (4 of 4 patients) than those with persistently low or undetectable virus load (<5000 genomes/ml) during the study period.
6.1.3 Blood CMV DNA in patients with CMV disease at baseline

All patients with CMV retinitis at baseline who had a tenfold or greater rise in CMV DNA blood levels had extraocular CMV disease, reactivation of retinitis, or both develop during follow-up. Conversely, some of the patients with reactivation of CMV retinitis had low/undetectable levels of CMV DNA. All five patients in whom extraocular disease developed, however, did have high or rising (greater than tenfold between successive visits) blood levels of CMV DNA before the onset of extraocular disease.

6.1.4 Future studies utilising quantitative CMV DNA load

Further study is needed to establish the optimum frequency with which virus load assays are performed to maximise their predictive value. Additional studies may indicate a role for CMV DNA blood levels in planning preemptive therapy before changes in CMV retinitis lesions become apparent clinically. A larger cohort study may also give some information as to the effect of CMV load relative to HIV load in predicting mortality and morbidity. Spector and co-workers\(^7\) have suggested that CMV load may be a predictor of survival in HIV infected individuals independent of HIV load, however, this has not been supported to date by published data.

\(^7\) Presented at the CMV retinitis, 2\(^{nd}\) multidisciplinary workshop Yosemite, California, Feb 1999.
We did not study patients receiving local therapy, but CMV load could also play a role in monitoring such patients. Reactivation of CMV retinitis may or may not be associated with rising CMV DNA levels. Determination of CMV load may therefore have important implications for the choice between systemic and local therapy. Reactivation with low levels of CMV in the blood may imply either inadequate ocular drug levels or localised virus resistance, and supplemental local therapy alone may be sufficient to bring disease under control. On the other hand, high CMV levels in the blood may reflect reactivation of disease throughout the body that requires systemic therapy.

With the advent of HAART therapy, the incidence of CMV disease is decreasing in developed countries. Despite the introduction of HAART, some patients may develop CMV retinitis at relatively high CD4+ T-lymphocyte counts, and some patients may become 'HAART failures', and therefore at risk of developing CMV disease. It would be useful to identify those patients most likely to develop CMV retinitis. Cytomegalovirus load may be one way of predicting those at risk, although with an increasingly large cohort of patients on HAART therapy, and the probable frequency of CMV load testing required, this may be impractical. It would be useful to reserve frequent CMV load testing to the most select group of patients, such as those that have lost CMV specific cell mediated immunity. Future studies may look into combining the initial testing of CMV immunity followed by serial CMV load measurements, as the most efficient way of predicting CMV disease development.

In 'HAART failure' patients on long term specific CMV therapies, drug resistance may become an issue. It is possible that virus load may become a proxy measure for resistance to therapy. If anti-CMV drug levels are known to he adequate
then a high virus load would suggest resistance has developed. However, it is known that different CMV strains (with different drug sensitivities) may be present in certain organs of the same patient. This strain variation within the same patient may limit the usefulness of a systemic venous blood CMV load acting as a proxy measure of resistance for a specific organ, such as the eye.

6.2. Blood Rheologic Factors

6.2.1 Abnormalities in rheologic factors associated with the HIV Disease

The major finding of this study, the significant increase of polymorphonuclear cells (PMN) pore transit times for HIV-infected patients with CMV retinitis versus either HIV-infected subjects without CMV disease or HIV-negative controls, indicates that PMN are more rigid in individuals with both HIV infection and CMV disease. CD4+ T-lymphocyte count was observed to modulate this increase of PMN rigidity for HIV-infected patients. There was an inverse relation between CD4+ T-lymphocyte count and PMN pore transit time; PMN from HIV-infected subjects without CMV retinitis and with a count of >100/μl had a pore transit times similar to HIV-negative controls.

Patients with clinical CMV disease had a lower haematocrit (p=0.002) than those without CMV disease. Red cell aggregation and ZSR were found to be correlated with CD4+ T-lymphocyte count in HIV infected patients.
6.2.2 Future studies

A longitudinal study is necessary to further understand the relationship between PMN rigidity and activation and the development of CMV disease and ‘HIV-retinopathy’ as well as ‘HIV dementia,’ which have been associated with markers of microvascular disease in some studies. In vitro studies exploring the relationship between CMV infection of a particular cell type, its activation state and rigidity, would also be useful in the understanding of this relationship.

If a relationship is established between PMN rigidity and disease development, then therapy may potentially be targeted to reduce PMN activation and rigidity. Established drugs, such as pentoxifylline are known to alter PMN activation, and rigidity.

In the post-HAART era microvascular abnormalities have been seen in patients without CMV retinitis, and this may be a cause of ocular morbidity. Ischaemic maculopathy, and neovascular disease can develop in HAART responders, and the understanding of rheologic abnormalities may help us understand and develop treatments for these patients, who are otherwise functionally well apart from decreasing vision.
Techniques looking at leukocyte rigidity and other rheologic factors may also be applied to help the understanding and treatment of ocular vascular disease, such as diabetic retinopathy and retinal vasculitis.

6.3. Retinal Capillary blood flow

6.3.1 Validation of HRF

The coefficient of variation ranged between 9 and 27.3, mean variation in HIV-negative test subjects was 16.28, and 18.25 in HIV positive subjects. This variation may be due to physiologic fluctuations in regional capillary blood flow with time, or it may be due to variability in the measurement technique, or the apparatus per se. There was no clear trend to greater variability in a particular region, and the amount of variability in a given region did not vary significantly between HIV positive and negative individuals.

In the in vitro model flow measurement was found to be significantly correlated to target speed ($p=0.11$), but no linear association was found between flow and alteration in focus or alteration in haematocrit of the target. There was a flow value measure that was generated even for a moving target and this value was greater if the haematocrit was not zero. However there was little difference found between the flow values when the haematocrit was altered. A flow value was generated even for non-moving target and these values were greater for targets with red cells. This 'zero-threshold' value could be the result of Brownian motion of particles on the slide as the LDF makes a non-directional measure of flow and this may explain the higher
zero-threshold value with stationary slides with a blood smear that would contain more particles. The large zero threshold may cross sectional studies less reliable unless this factor could be taken into account

6.3.2 Abnormalities in retinal capillary flow associated with the HIV Disease

No statistically significant difference was found in flow measurements between the HIV positive and negative groups as well as between the HIV positive, CMV positive and HIV positive, CMV negative groups. This result may be reflect a true lack of difference in retinal blood flow between the groups or the study design may not have been able to detect a difference as the sample size may have been too small, or due to variability in measurements. In addition there is a possibility that retinal capillary blood flow could vary with different stages of HIV-related disease, as blood flow in diabetics varies with different stages of diabetic retinopathy, and this could explain the lack of expected association.

6.3.3 Potential and future applications of measuring retinal capillary blood flow

Further studies subgrouping HIV infected patients and also investigating autoregulation of retinal capillary blood flow may be more informative, as blood flow may vary with different clinical disease states. Other methods of measuring retinal capillary flow, such as the blue-light entoptoscope may be useful in clinical studies, as this technique specifically looks at leukocyte movement in the macula capillaries and this could then be related to in vitro measurement of PMN rigidity.
6.4 Concluding remarks

Despite the fact that the incidence of CMV retinitis has dropped in association with protease inhibitor use, there is still a need for tests that can identify patients at most risk of developing CMV disease or in the pre-clinical stages of infection; it could be argued that it is even more important now to have better tools that will identify the specific, smaller subset of patients still at risk for CMV disease and its complications, thereby avoiding unnecessary use of expensive and toxic anti-CMV drugs in the larger population. Quantitative measures of CMV load and measurement of certain rheologic parameters in the peripheral blood have potential clinical application in identifying patients at great risk of developing active CMV disease.
7 References


Collaborative DHPG Treatment Study Group. (1986) Treatment of serious  
cytomegalovirus infections with 9-(1,3-dihydroxy-2-propoxymethyl)guanine  
in patients with AIDS and other immunodeficiencies. N.Engl.J.Med. 314,  
801-805.

Collier, A.C., Meyers, J.D., Corey, L., Murphy, V.L., Roberts, P.L. and Handsfield,  
sexual practices, antibody to human immunodeficiency virus, and cell-  

and Santos, G.W. (1994) The eye in bone marrow transplantation. VI. Retinal  

Pathol 18, 525-542.

with normal eyes and with acute cytomegalovirus chorioretinitis.  
Am.J.Ophthalmol. 80, 817-824.

value of CD4 lymphocyte numbers for the development of opportunistic  
infections and malignancies in HIV-infected persons.  

D’Amico, D.J., Skolnik, P.R., Kosloff, B.R., Pinkston, P., Hirsch, M.S. and Schooley,  

endothelium in AIDS. Ophthalmologica 197, 169-175.

(1990) Localization of human cytomegalovirus in peripheral blood leukocytes  

and reproducibility of bidirectional laser Doppler velocimetry for the  

(1990) Therapy for cytomegalovirus polyradiculomyelitis in patients with  


Serum antibodies to individual cytomegalovirus structural polypeptides in 
renal transplant recipients during viral infection. *Microbiol. Immunol.* 30, 
683-695.

cytomegalovirus structural polypeptides during primary infection. 

delayed specimen processing on cytomegalovirus antigenemia test results. 

Lang, D.J., Ebert, P.A., Rodgers, B.M., Boggess, H.P. and Rixse, R.S. (1977) 
Reduction of postperfusion cytomegalovirus-infections following the use of 
leukocyte depleted blood. *Transfusion* 17, 391-395.

Leach, C.T., Detels, R., Hennessey, K., Liu, Z., Visscher, B.R., Dudley, J.P. and 
Cherry, J.D. (1994) A longitudinal study of cytomegalovirus infection in 
human immunodeficiency virus type 1-seropositive homosexual men: molecular 
epidemiology and association with disease progression. *J. Infect. Dis.* 170, 
293-298.

Levin, A.V., Zeichner, S., Duker, J.S., Starr, S.E., Augsburger, J.J. and Kronwith, S. 
(1989) Cytomegalovirus retinitis in an infant with acquired 
immunodeficiency syndrome. *Pediatrics* 84, 683-687.

cytomegalovirus by enzyme-linked immunosorbent assay (ELISA). 

Lichtman, M.A. (1973) Rheology of leukocytes, leukocyte suspensions and blood in 

Ljunggren, H.G. and Kserre, K. (1990) In search of the 'missing self': MHC 
molecules and NK cell recognition. *Immunol Today* 11, 237-244.

CD8+ T lymphocytes and CMV retinitis in patients with AIDS. *Am J 
Ophthalmol* 120, 283-290.

Lowe, G.D. (1981) Report on working group meeting: red cell deformability-

636.


8.0 Appendix

8.1 Background to Laser Doppler Flowmetry

The measurement of blood flow by scanning Laser Doppler Flowmetry (SLDF) is based on the optical Doppler effect: laser light scattered by a moving particle is shifted in frequency by an amount $\Delta f$, as shown in Equation. 1.

$$\Delta f = (1/2\pi) (k_{sc} - k_i) \cdot v.$$  \hspace{1cm} (1)

Where $k_{sc}$ is the wave vector of the scattered light, $k_i$ is the wave vector of the incident light and $v$, the velocity vector of the moving particle. When the laser beam impinges on a tissue containing moving blood cells the spectrum broadens. It has a width that depends on the velocity vector and the scattering in the tissue itself. The collected light is guided to a photodetector and analysed using the method described by Bonner and Nossal. (Bonner and Nossal, 1990)

The effective Doppler shift, called "flow," is calculated by Eq. 2.

$$\omega = 2\pi \sum_{125 Hz}^{2000 Hz} fP(f) df / P(f = 0)$$  \hspace{1cm} (2)

where $P(f)df$ is the power of the photodetector current associated with fluctuations in a frequency range $df$ about $f$(Hz) and $P(f = 0)$ is the average power (DC power) in the detected signal. "Flow" describes the distance gone by all moving cells inside the sample volume per unit time. Frequencies lower than 125 Hz are
excluded to minimise the influence of low-frequency artefacts such as breathing, cardiac action, and intensity fluctuations due to other mechanical effects.

The fraction power of the Doppler shift, called 'volume' is calculated as shown in Eq.

$$\omega = 2\pi \sum_{125 \text{ Hz}}^{2000 \text{ Hz}} fP(f)df / P(f = 0)$$  \hspace{1cm} \text{(3)}$$

"Volume" ($\chi$) is proportional to the mean number of photon collisions with moving cells, i.e., to the number of moving red blood cells (RBC) in a sample volume of tissue. The value of $X$ can be used to determine the concentration of blood cells moving in the tissue.

The mean RBC speed, called "velocity," can be calculated from Eq. 4.

$$\text{Velocity} = \omega / \chi$$  \hspace{1cm} \text{(4)}$$

From the calculated data of flow (Eq. 2), volume (Eq. 3), and velocity (Eq. 4), two-dimensional maps of the retinal perfusion are created.
8.2 Publications and presentations arising from this work

**Papers**


2) A.Tufail, Holland G.N, Fisher T.C. Meiselman H.J. Decreased polymorphonuclear leukocyte deformability in HIV-infected individuals.(submitted 1999)

**Published abstracts**


Presentations


5) Association for Research in Vision and Ophthalmology Annual Meeting

6) Association for Research in Vision and Ophthalmology Annual Meeting