THE SIGNIFICANCE OF HUMAN CYTOMEGALOVIRUS INFECTION IN HEALTHY BLOOD DONORS

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ABSTRACT

Human cytomegalovirus (HCMV) infection has been studied in blood donors. The subsequent establishment of latency by the virus is a significant factor in blood transfusion practice as the latent virus may be transmitted by transfusion to susceptible patients with serious consequences. The current prevalence of HCMV-IgG in the donor population was found to be 47% and related to donor age and sex; increasing with age and significantly higher in females (p < 0.001). The prevalence of HCMV-IgM and -IgA in the donor population was determined in an attempt to identify recently infected donors, purported to give rise to an increased incidence of HCMV transmission. They were detected in 0.09% and 1.5% of donors respectively; no other indications of recent infection were detected. The immune response to HCMV was found to be stable with respect to Ab titre and immunoblotting profiles. The current seroconversion rate of HCMV-Ab negative donors was found to be approximately half of that expected.

To identify acutely infected donors and to confirm the presumed sites of latency, studies were performed using conventional and molecular techniques to detect either infectious virus or viral nucleic acid in donated blood or saliva and urine samples obtained from donors. HCMV nucleic acid was not detected in serum or leucocytes by dot-blot hybridisation, although HCMV DNA and IE RNA were demonstrated in leucocytes using the PCR technique. Urine from one seropositive donor was found to contain detectable HCMV by cell culture and PCR. No saliva samples were found to contain HCMV. Although HCMV can be detected in healthy donors using molecular techniques, HCMV-Ab screening remains the most appropriate method for determining those donations likely to transmit HCMV.
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<td>Natural killer (cells)</td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell(s)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline (Dulbecco's)</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming units</td>
<td></td>
</tr>
<tr>
<td>PMNL</td>
<td>Polymorphonuclear leucocytes</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>Post transfusion</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
<td></td>
</tr>
<tr>
<td>RIST</td>
<td>Radioimmunosorbent test</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase/transcription</td>
<td></td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
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The increasing use of blood transfusion, particularly component therapy, has led to heightened awareness of the significance of transfusion transmitted diseases. This has been highlighted in recent years by the emergence of AIDS and the role of blood transfusion as a potential route of infection for the human immunodeficiency virus (HIV). However, it has been known for many years that a number of other viral infections may be transmitted by transfusion. One such virus is human cytomegalovirus (HCMV), recognised for more than 20 years as a potentially serious complication of blood transfusion in certain patient groups.

HCMV usually infects asymptomatically, but it can produce serious infections in immunocompromised individuals, notably the very young and the immuno-suppressed. The clinical importance of HCMV has increased in step with the increasing use of transplantation and consequent immunosuppression in many areas of modern medicine, together with the increase in the number of neonatal care units and their ability to support increasingly premature infants.

Blood transfusion is an important route of HCMV transmission, because the virus exhibits persistent latency after initial infection and the site of latency is thought to be the circulating mononuclear white blood cells (PBMC). Numerous studies have demonstrated the link between transfusion and HCMV infection and have further shown that the use of blood from donors without circulating Ab to HCMV greatly decreases the risk of post transfusion HCMV infection.
CHAPTER 1 INTRODUCTION

1.1 HISTORY

The first report of HCMV infection was made in 1904 when Jesionek and Kiolemenoglou identified 'protozoan-like' cells in the lungs, kidney, and liver of a stillborn, eight month syphilitic foetus. Ribbert (1904) reported finding similar cells 23 years previously in a similar foetus and in the parotid glands of children. Over the next few years further similar reports appeared. Goodpasture and Talbot (1921) first challenged the idea of protozoan infection after finding intranuclear inclusions similar to those previously described in varicella infection (Tyzzer 1906). Lange (1922) and Mueller (1922) both reported similar inclusions in the kidneys of infants and surmised that the finding of these inclusions in stillbirths ruled out protozoan infection, because protozoa would not have passed the placental barrier. In 1925 Von Glahn and Pappenheimer (1925) described the first case of an adult with a liver abscess that contained similar inclusions which they concluded were caused by a virus identical or closely related to viruses in the herpes family.

The first conclusive evidence for the viral basis of the disease was reported by Minder (1953) who showed 100nm particles in cytomegallic inclusion cells from the pancreas of a 14 day old infant. Subsequently Rowe et al (1956), Smith (1956), and Weller et al (1957), isolated cytopathogenic viruses that produced the cytomegallic inclusions characteristic of the, then termed, human salivary gland virus.

Inclusion of the virus in the group of herpesviruses was mainly on the basis of morphology (Wright et al, 1964), all members of the herpesvirus group identified so far having the same unique
morphology. The group is now divided into three subfamilies, α- (herpes simplex viruses), β- (cytomegaloviruses), and γ- (lymphoproliferative viruses). This division is based upon genome organization, growth rate, host range, degree of cell association of virus in culture, and antigenicity.

1.2 EPIDEMIOLOGY OF HCMV INFECTIONS

1.2.1 Congenital Infections

HCMV is the most common viral infection to be transmitted in utero, with an incidence ranging from 0·2% to 2·2%, of all live births throughout the world (Stagno et al., 1981a), and an incidence of 0·3 to 0·4% in the UK (Peckham et al., 1983). The virus from the infected mother crosses the placenta and directly infects the foetus. Primary infection is usually asymptomatic (Griffiths et al., 1980), and symptomatic infections generally present as a mononucleosis-like syndrome.

Although primary infection of the mother is associated with a higher risk of congenital disease, recurrent infections give rise to a higher incidence of congenital infection (Stagno et al., 1977; Schopfer et al., 1978; Stagno et al., 1982). Recurrent infections are rarely symptomatic, though excretion of virus can be demonstrated and a rise in IgG titre may also be found.

Although infection is common, the incidence of clinical disease is low. Most infected infants (90–95%) are asymptomatic at birth, and in the main suffer no adverse effects. However, the outcome of infection in symptomatic individuals can be serious, and infection of the central nervous system is an important cause of handicap (Mellish & Hanshaw,
1973). Of the 5 to 10% of infants who are symptomatic no more than 2% have severe disease with CNS involvement, the remainder usually have evidence of extraneural infection, consisting of hepatosplenomegaly, anaemia, pneumonitis and jaundice.

Unlike some other intrauterine infections, infection with HCMV can and does occur in women already HCMV-Ab positive before conception. It is now known that HCMV-Ab positive women are prone to reactivation during pregnancy despite the presence of circulating Ab (Stagno et al, 1977).

Whilst most studies have found that infants infected as a result of recurrent maternal infection are asymptomatic at birth and do not develop clinical disease, a number of cases of severe symptomatic disease have been reported in such infants. Ahlfors et al (1982) reported the case of an infant, congenitally infected by recurrent maternal infection, with sensorineural hearing loss; Peckham et al (1983) similarly identified two such infants, one with severe neurological damage and sensorineural hearing loss, and the other with recurrent HCMV pneumonitis during infancy.

1.2.2 Perinatal Infection

HCMV infections acquired during the first few months of life are referred to as perinatal infections. Diagnosis is generally made on the basis of laboratory findings, infection being characterised by the absence, at birth, of HCMV-IgM in the blood and of virus in the urine. In most cases, virus excretion begins within 10 weeks and may persist for over a year; Ab levels may rise accordingly.
The majority of such infections are subclinical and undiagnosed, and
the infants develop normally (Granström et al, 1977). Infants who are
preterm, of low birth weight, suffering from respiratory distress
syndrome, and requiring prolonged hospitalisation and intensive care
seem to be at highest risk of clinically significant perinatal infection
(Ballard et al, 1979; Yeager et al, 1981). Infection may result from
exposure to virus excreted in the cervical secretions during delivery
(Reynolds et al, 1973), direct transmission in infected breast milk
(Stagno et al, 1980), nosocomial spread from other infected individuals
(Spector, 1983), or by blood transfusion (Yeager et al, 1981).

Cabau et al (1979) demonstrated a close association between the
serological status of the mother and acquisition of infection by the
infant in the first year of life, suggesting that the most important
source of infection, or of protection from infection, may be the mother.
The presence of passively acquired maternal Ab, does not, however,
prevent perinatal infection (Adler et al, 1983; Yeager et al, 1983; de
Cates et al, 1988), although in a number of cases this may have been
due to the decline in level of maternal Ab (Yeager et al, 1983). de
Cates et al (1988), however, reported a case of fatal perinatal HCMV
infection due to blood transfusion of an infant born of a HCMV-Ab
positive mother, despite the presence of transplacentally acquired
maternal Ab at the same titre as at birth.

Infection of mothers during pregnancy may lead to perinatal rather
than congenital infection. Kumar et al (1975) found that 20% of HCMV
negative infants born to mothers with cytomegaloviruria during
pregnancy, became viruric before 11 weeks of age. Horizontal
transmission of virus between infected and uninfected infants has also
been demonstrated, but the frequency and significance of this route is
uncertain. Spector, (1983), used restriction enzyme analysis of isolates from infected infants to determine the source of transmission, and Gurevich & Cunha (1981) documented the case of an infant with fulminating congenital HCMV infection who, although she only lived for eight hours, infected four other infants in an intensive care unit. However, other studies have concluded that horizontal transmission occurs infrequently if at all (Adler et al, 1986; Yeager et al, 1983). Adler (1986) also found by restriction enzyme analysis that 34 isolates from infected infants in care units were all different.

Perinatal infection due to HCMV in sick neonates gives rise to a recognisable disease state which appears to have a predictable course and to be self limited. The most common source of infection is either exposure at birth to virus excreted from the cervix or transmission by blood transfusion.

1.2.3 Infection In Immunocompetent Individuals

Infections in immunocompetent individuals are usually asymptomatic. When symptoms appear, they most commonly manifest as a heterophile-antibody-negative, mononucleosis syndrome which commonly includes; fever, myalgia, atypical lymphocytosis, mild hepatitis, splenomegaly, pharyngitis, and pneumonitis. Although this syndrome is predominantly found in adults, it does also occur in childhood (Ho, 1982).

1.2.3.1 Infection in Childhood

Infection in childhood is difficult to study because of the high incidence of asymptomatic infections. Detection of excreted virus in saliva and/or urine is used to diagnose infection, although excretion does not necessarily indicate acute infection and may stem from
persistence of a previous infection. Transmission between individuals in younger age groups probably occurs by prolonged and repeated exposure to virus shed from other children, for example Hutto et al (1986) isolated virus on the toys and hands of children attending day care centres.

1.2.3.2 Infection in Adulthood

Infection in adults is also difficult to study for similar reasons. The precise modes or frequency of transmission of infection amongst adults are not known, though it would seem most likely that spread is by direct contact with infectious virus in body secretions (Adler, 1986).

Nosocomial transmission has been studied by a number of workers, specifically involving nursing staff in renal or neonatal units (Balfour & Balfour, 1986; Dworsky et al, 1983; Young et al, 1983). These studies, however, have been unable to show any significant difference between the infection rates in the nursing staff and control groups, even in situations where staff were exposed to a high incidence of viral excretion.

Evidence for venereal transmission has been found in homosexual men (Mintz et al, 1983; Ikram et al, 1983; Embil et al, 1986), in whom a high incidence of HCMV infection has been detailed as well as high titres of HCMV specific Ab. The prevalence of HCMV infection was found to be significantly higher in homosexual men than in a control group of heterosexual men. The finding of intermittent HCMV-specific IgM Ab in infected individuals (Mintz et al, 1983) may indicate frequent reactivation of latent infection or frequent exposure to virus. The shedding of virus in semen is common and presumably central to the spread of infection (Buimovici-Klein, 1988). It is possible that repeated
exposure to virus could result in an extended state of immunosuppression, and if this were the case, such a state might then play an important part in triggering AIDS in these HIV infected individuals. Venereal transmission in heterosexual individuals has been reported (Demmler et al. 1986).

A further route of infection has been described by Schmitz et al. (1985), who found an increase in the rate of primary HCMV infection in previously healthy adults returning from tropical areas. In countries with a higher prevalence of HCMV infection exposure to shed virus must necessarily be more frequent. Seronegative individuals visiting these countries are thus at greater risk of infection.

Though transmission by blood transfusion is an important source of infection, it must be an incidental route in the spread of the virus through the general population given that most individuals do not undergo transfusion during their lifetime, and its significance is difficult to assess. Furthermore, to assess the real risk of transmission by transfusion, infection by exogenous virus via more natural routes must first be considered and ruled out.

Transmission among healthy adults probably accounts for the majority of infections in developed countries and may result from a combination of all the routes discussed above.

1.2.4 Infection In Immunocompromised Individuals

HCMV infection is a common finding following immunosuppression which may be therapeutically induced or as a consequence of a particular disease state. The mode of infection is not clear because of the possibility of reactivation of a latent infection. In general, secondary
(recurrent) infection gives rise to less severe disease than does primary infection (Glenn, 1981).

1.2.4.1 Infection Associated With Clinical Immunosuppression

Pathological causes of immunosuppression are many and varied and all may lead to primary or secondary infections with HCMV. Probably the most important clinical condition currently associated with severe HCMV infection is AIDS, where HCMV disease is a major cause of mortality and morbidity, and which usually manifests as HCMV pneumonitis (Quinnan et al., 1984; Niedt & Schinella, 1985). It is also thought that HCMV, rather than being merely an opportunistic infection, may mediate the disease state in AIDS patients through its own ability to cause immunosuppression (Drew et al., 1985).

1.2.4.2 Infection Following Therapeutic Immunosuppression

Sutton et al. (1971) reported an increased incidence of HCMV infection in children with leukaemia following treatment with cytotoxic drugs. The resulting disease was either mild or asymptomatic and infection did not adversely affect the prognosis. This compares with adult leukaemias, where HCMV infection can be serious, leading to disseminated infection and pneumonitis.

Immunosuppressive therapy is essential to successful transplantation programmes, but has the unfortunate side effect of increasing the susceptibility of the individual to certain viral infections, most notably, HCMV. HCMV infection is the most common cause of death among transplant recipients, and is the most common cause of serious infection in the early post-transplant period. A review by Ho (1982) established that 52-80% of transplant patients became infected with
HCMV. It was further apparent that in essentially all HCMV-Ab positive patients (>85%) latent infection reactivated after transplantation, whilst only 53% of HCMV-Ab negative patients developed primary infection.

In cases of primary infection, the most common source of virus is the transplanted organ. Ho et al (1975) demonstrated infection in 83% of HCMV-Ab negative recipients given kidneys from HCMV-Ab positive donors but in only 30% of HCMV-Ab negative recipients given kidneys from HCMV-Ab negative donors. This was further supported by similar reports from Betts et al (1975) and Tobin et al (1979).

In secondary infections, the source of virus could be reactivation of endogenous virus, or exogenous virus. Both Chou (1986) and Grundy et al (1986) have shown that HCMV-Ab positive recipients can be infected with exogenous virus from the transplanted kidney. Recently, Grundy et al (1988) have further demonstrated that reinfection by virus in the donor kidney is more significant than reactivation of latent virus. Symptomatic infection was found to occur more frequently upon reinfection than following reactivation.

Wreghitt et al (1988) studied the first 166 heart and heart/lung transplants at the Papworth hospital (Cambridge, UK), and found that 83% of HCMV-Ab negative recipients receiving a heart from an HCMV-Ab positive donor acquired primary HCMV infection, and that 20% of these subsequently died. Gorensek et al (1988), however, have shown that donor serological status is not as significant as steroid dose in the severity of post-transfusion infection of heart transplant recipients. They showed that only high steroid dose was associated with clinically significant HCMV disease, although if the total HCMV infection rate is considered, both steroid dose and donor serology
were found to be significant. In a subsequent report Wreghitt (1989) gave some support to these findings. Although it was found that all deaths from primary infections were associated with transplantation of a HCMV-Ab positive heart to a HCMV-Ab negative donor, the severity of disease correlated with the degree of immunosuppression.

HCMV is the infectious agent which most commonly causes death following allogeneic marrow transplantation, largely due to HCMV pneumonia which is fatal in 85% of cases (Meyers et al, 1983). Pneumonitis occurs most commonly in patients undergoing allogeneic transplantation for leukaemia or other malignancies with an incidence of 15% (Meyers et al, 1986). The risks of HCMV infection are compounded by the use of high dose radiation and cytotoxic drugs, and by the fact that most patients receive large volumes of blood, often untested for HCMV. Meyers et al (1986) studied 545 marrow transplant patients and found evidence of HCMV infection in 51.4%; infection occurred in 36% of HCMV-Ab negative and 69% of HCMV-Ab positive patients.

The incidence of HCMV infection in liver transplantation is less than with other organs, and is estimated at 1 to 15% (Singh et al, 1988; Paya et al, 1989a). Paya et al, (1989b) reported a study of 93 transplants in 78 patients, in which 26% of patients acquired HCMV infection. Infection patterns were similar to those seen in other organ transplant recipients (Singh et al 1988).

The incubation period for post-transplantation HCMV infection appears to be remarkably constant and is usually one to four months (Ho, 1982), the incubation period of primary infections being slightly shorter (2 months) than that of secondary infections (3.5 months) (Ho et al,
Infections are frequently asymptomatic, the incidence of symptomatic infections varying from 30 to 55% of cases among all groups of transplant patients. Symptomatic infections vary from the febrile mononucleosis syndrome described previously, which appears to be very common in transplant patients, to disseminated infection with pneumonitis and in some cases CNS involvement.

HCMV is clearly an important pathogen in immunocompromised patients. Although our understanding of many aspects of the disease process has increased in recent years, further work is required to elucidate the mechanisms involving latency, especially reactivation of latent virus in immunocompromised individuals.

1.3 PHYSICAL CHARACTERISTICS

1.3.1 Virus Structure

There are more than 70 herpesviruses and they infect a wide range of hosts. Although their growth characteristics are diverse, they are closely linked by morphological and molecular homology. The structure of the HCMV virion is characteristic of the herpesvirus genus (Roizman, 1982). HCMV is the largest member of the herpesviruses, and has the largest genome and most complex host interrelationship, of any known virus.

The genome is complexed with protein to form the core and this is encased in an icosohedral capsid, constructed from 162 capsomeres, each consisting of one or more protein molecules. The capsid is 100nm in diameter and is itself enclosed by the tegument, which is further bounded by an outer lipid bilayer (envelope) containing protruding
viral proteins, resulting in a final diameter of approximately 180nm for the whole virion (Wright et al, 1964; Kanich and Craighead, 1972).

More detailed studies of the structural and antigenic features of the virus led to the description of a second structural form of HCMV which is produced during culture of the virus in vitro (Craighead et al, 1972). These particles, termed dense bodies, were found to be antigenically similar to the virion but morphologically distinct, and to contain electron dense material bounded by an outer membrane.

The isolation and identification of a third particle was reported by Irmiere & Gibson (1983). This particle was found to be morphologically and antigenically similar to the virion, except that it contained no DNA core. It was subsequently termed a non-infectious enveloped particle (NIEP).

During virus replication, proteins synthesised in the cytoplasm are first assembled within the nucleus to form the A capsid. Further proteins are added to form the B capsid, and finally DNA is incorporated to form the C capsid. Excess 69 kDa protein not incorporated into B capsids is enveloped and released in the form of dense bodies. Completed C and excess B capsid both leave the nucleus and pass into the golgi apparatus where they are released as virions and NIEP's respectively (Figure 1.1). Lee et al (1988) found that B capsid could still be produced in the absence of DNA synthesis.
Figure 1.1 Schematic outline of assembly of HCMV particles (from Griffiths & Grundy, 1987)

[numbers represent the approximate MWs of proteins]

1.3.2 Molecular Biology

HCMV DNA was first isolated by Crawford & Lee (1964), and further characterised by Huang et al (1973). Structurally, it resembles the genomes of other herpesviruses, although it is significantly larger (De Marchi et al, 1978). The genome is a linear, double stranded DNA molecule of high complexity, having a molecular weight (MW) of 150 x 10^6. Recently the complete sequence of HCMV has been determined (Chee et al, 1990), which reveals a length of 229,354bp and 57.2% G+C content, the theoretical coding capacity being approximately 200 proteins. The genome is composed of segments of repeated and
unique sequences (LaFemina & Hayward, 1980), the unique sequences being found in areas of the genome designated long and short (UL and Us) (Figure 1.2). These sequences are separated by short repeat sequences and can be orientated in either direction, resulting in four isomeric forms of the genome which are usually found in equal proportions (Ebeling et al, 1983; Westrate et al, 1980; Oram et al, 1982; Fleckenstein et al, 1982; DeMarchi, 1981).

Genomic variation among herpesviruses was first studied by Huang & Pagano, (1974), Huang et al (1976), and Kilpatrick et al (1976), who showed little or no homology between HCMV DNA and CMV DNAs of other species, and minimal (5%) homology between HCMV and other human herpesviruses. Efstathiou et al (1988), have demonstrated areas of homology between the recently described human herpesvirus 6 (HHV-6) and HCMV.

![Restriction maps and schematic diagram](image)

**Figure 1.2** Hind III and Eco RI restriction maps of AD169 DNA and schematic diagram of the four isomeric forms of the DNA. L, long segment; S, short segment; U, unique segment; I, internal repeat; T, terminal repeat.
Minson & Darby (1982) found an overall homology of approximately 80% between different strains of HCMV, although it is not known whether there are regions which show more or less homology. When the restriction patterns of strains of HCMV were studied (Kilpatrick et al., 1976), certain co-migrating bands were found to be common to all strains, though no two strains gave the same profile. When similar restriction maps were produced for the HCMV reference strains Towne, AD169 and Davis, it became apparent that some fragments have the same size and map positions in the three strains. This led LaFemina & Hayward (1980) and Westrate et al. (1983) to propose the collinearity of HCMV genomes, and this was confirmed by Colimon et al. (1985) using cloned fragments of the AD169 strain of HCMV (Fleckenstein et al., 1982).

The establishment of heterogeneity between the genomes of different strains of HCMV, (Huang et al., 1976; Grillner & Blomberg, 1984; Tyms, 1983; Garrett & Warren, 1985), has facilitated the identification of individual strains and subsequent epidemiological studies (Spector, 1983; Toplin et al., 1985; Chou, 1989b; Kanesaki et al., 1989). The differences most commonly detected were the presence or absence of restriction cleavage sites, and some variability at the Ul and Us junctions (Westrate et al., 1983). By screening Southern blots with cloned AD169 DNA probes, Chou et al. (1984), showed that all genomic regions from 21 isolates of HCMV hybridised identically. The DNA sequences of HCMV strains are therefore well conserved, but limited divergence, probably at the level of single base substitutions, results in the observed restriction enzyme fragment length polymorphisms.

Peden et al. (1982) first demonstrated homology between HCMV DNA and mammalian cellular DNA, and Rüger et al. (1984) and Shaw et al. (1985) have identified five regions of cell-related sequences in the genome of
strain AD169. There is no evidence that HCMV DNA has any homology with the proto-oncogene c-myc or the avian retrovirus oncogene v-myc (Ruger et al., 1984; Rasmussen et al., 1985b).

The replication of HCMV DNA has not been widely studied, but the similarity in genome structure of HSV and HCMV may indicate that the rolling circle model for HSV replication, proposed by Jacob et al. (1979), may apply to HCMV. Concatemers of viral DNA are cut to genome length and encapsidated. These concatemers, and also circular forms, are produced in abundance in permissive cells during the first 48 hours post infection (LaFemina & Hayward, 1983).

1.3.2.1 Transcription and Translation

Knowledge of early virus gene expression is important for understanding virus/cell interactions, especially in the case of HCMV where latency may result from a defect in early gene function. Following infection of a permissive cell, expression of the HCMV genome occurs in a temporally regulated cascade (Stinski, 1978; Wathen et al., 1981). RNA transcripts and proteins are produced in three main phases termed immediate early (α), early (β) and late (γ).

After entry of the virus into the host cell, the immediate early (IE) genes are the first to be transcribed by host cell RNA polymerase II (Thomsen et al., 1984; Spaete & Mocarski, 1985). Following their expression, one or more as yet uncharacterized α gene products transcriptionally transactivate the β and some γ genes (Geballe et al., 1986). Once this activation has occurred, there is a delay in full expression of β and γ gene products due to posttranscriptional regulatory events (Geballe et al., 1986).
Figure 1.3 shows a simplified pattern of the expression of the HCMV genome, and the effect of inhibitors of protein and DNA synthesis.

The use of cycloheximide to block translation of mRNA without inhibiting transcription has shown that IE transcripts can be produced in the absence of de novo protein synthesis (Stinski et al., 1983; Wathen & Stinski, 1982). IE RNA has been shown to be produced from only a few defined regions, comprising 20% of the genome. The main area of transcriptional activity has been located at a common position between map units (mu) 0.66–0.77 in all HCMV genomes so far studied, and four major transcription units (IE1–IE4) have been identified (Jahn et al., 1984; Stinski et al., 1983). The IE proteins are regulatory proteins which accumulate in the nucleus and are also

\[ \text{cx: cycloheximide; AD: actinomycin D; PAA: phosphonoacetic acid; Ara-C: cytosine arabinoside; REP: hypothetical site of origin of replication; +: stimulatory effect; [solid line]: metabolic block} \]
responsible for changes in the host cell chromatin structure and template activity (Kamata et al, 1978, 1979).

After IE proteins have been produced a switch from restricted to extensive transcription of the genome takes place, presumably mediated by the IE proteins, and β-mRNA is produced. IE1 is referred to as the major IE gene and is transcribed to the most abundant species of IE RNA, 1·95kb in size (Wilkinson et al, 1984; Jahn et al, 1984). The protein encoded by this mRNA is the predominant IE protein found in cells within one hour of infection (Stenberg et al, 1984), and has a MW of 63·8 kDa (Akrigg et al, 1985). Other proteins are also produced at low levels during the IE period, and their precise functions have yet to be clearly defined. During the second (β) phase of gene expression approximately 75% of the genome is transcribed. Two major classes of mRNA, 1·2 and 2·7kb, have been shown to be transcribed from the long repeat regions during this period (McDonough et al, 1985). The functions of most of the proteins produced have not been elucidated, although some play a part in the induction of host cell macromolecular synthesis and the development of cytopathic effects (Furukawa et al, 1973, 1975a; Tanaka et al, 1975).

The late period (γ) follows the onset of viral DNA synthesis, when more than 90% of the genome is transcribed into stable RNA's which then become associated with the polyribosomes (DeMarchi, 1984). Many proteins and glycoproteins are synthesised during this period (Landini & Michelson, 1988), most of these 'infected cell-specific proteins' (ICSPs) are coded by the virus, although some may be virus-induced, host proteins.
1.3.3 HCMV Proteins

The size and complexity of the HCMV genome is such that it is theoretically capable of coding for over 200 proteins. Landini & Michelson (1988) have reviewed over 100 papers that have appeared in the last 10-12 years, and that have described HCMV proteins of over 140 apparently different molecular weights. The virion itself is estimated to consist of 30-35 different structural proteins (Rasmussen et al, 1984).

Host cell protein synthesis is stimulated by HCMV infection. After virus adsorption, cellular protein synthesis is inhibited for a short period (Garnett, 1979), and then increases dramatically (Furakawa et al, 1973) with the result that viral protein synthesis during the early phase of infection represents only 30% of total protein synthesis (Stinski, 1978). Even at the peak of viral protein synthesis, no more than 60% of the nascent protein is virus-specific (Stinski, 1978). Furthermore, analysis of purified virions (Mar et al, 1981; Landini & Michelson, 1988) has shown the presence of host cell proteins. These findings have all complicated investigations because of difficulties in determining which proteins are virus specific and which are virus-induced host cell proteins. Classification of the HCMV proteins is primarily based on whether or not the protein is structural. The proteins can, however, also be described as α, β, or γ according to the transcription and translation cascade of genome expression as described previously (most structural proteins are γ proteins, whilst the nonstructural proteins are α and β proteins).
1.3.3.1 Structural Proteins

Structural proteins were initially thought to be synthesised only after viral DNA synthesis, though Mocarski et al. (1985) have found at least one that is synthesised before DNA synthesis, and more may eventually be described. Many of the structural proteins in the tegument are phosphorylated, unlike those in the envelope which are usually glycosylated. Landini & Michelson (1988) studied and classified according to molecular weight 113 structural proteins described in the then current literature. This study yielded 55 MW size groups, with 32 glycopolypeptides and 23 phosphopolypeptides described. The number of size groups may appear to be high for a number of reasons: although SDS-PAGE gives good resolution of proteins, 2-D gel analysis of virion proteins has shown that any given band in the gel may actually contain several polypeptides (Haarr & Marsden, 1981; Roby & Gibson, 1986), the purified preparations may contain proteins in various stages of processing, host-cell virus-modified proteins may be present, different researchers assign different MWs to the same proteins, and there are both glycoproteins and phosphoproteins of the same MW in five of the groups described.

Purified virions of HCMV contain three main phosphoproteins (150, 71, 65 kDa), and at least nine other minor proteins of various size (Roby & Gibson, 1986). NIEP's also contain these proteins together with one extra major protein, the 36 kDa assembly protein. Coding regions for the proteins of 150 (Jahn et al., 1987), 71 (Ruger et al., 1987), 67 (Davis & Huang, 1985), 65 (Ruger et al., 1987), and 28 kDa (Meyer et al., 1988) have been identified. None of the phosphoproteins show homology at
the amino acid level to reported sequences from other human
herpesviruses.

Glycoprotein structure is of interest in the investigation of the
immune response to HCMV, and the development of HCMV-specific
subunit vaccines. Little is known about protective responses to HCMV,
and the plethora of structural and nonstructural proteins encoded by
the virus has hindered identification of target antigens for the
induction of potentially protective responses. Studies have implicated
viral glycoproteins as the most likely targets of protective responses,
based on their immunogenicity (Hayes et al, 1987; Pereira et al, 1982a),
the presence of several readily detectable glycoproteins on the
surface of infected cells, and the fact that protective immunity to
many viruses, including other herpesviruses, is directed against
envelope glycoproteins.

Investigation of glycoproteins is hindered by their low abundance in
virions and the formation of complexes. Several disulphide linked
complexes have been identified in the envelope of HCMV virions or
dense bodies (Britt, 1984; Farrar & Greenaway, 1986; Rasmussen et al,
to describe three groups of glycoproteins, gcI (gp55 and gp93-130
kDa), gcII (gp47-52 kDa) and gcIII (gp86 and gp145 kDa). Monoclonal
antibodies (MoAbs) as well as monospecific sera, specific for gcI, are
capable of neutralizing HCMV in the presence or absence of
complement (Britt, 1984; Kari et al, 1986; Rasmussen et al, 1985a, 1988;
positive adults and congenitally infected children are able to mount a
B-cell response to proteins of the gcI and gcII complexes.
A gene family, HXLF, which consists of five genes that share regions of homology and are arranged in tandem, lies in the short unique region of the genome (Weston & Barrell, 1986). Gretch et al (1988a) showed that at least two of these genes (HXLF1, HXLF2) encode proteins of 20-25 kDa, which are glycosylated to 47-52 kDa, and complex with one another and other members of the family to make up the complex gcII. A characteristic of this complex is diffuse migration in SDS-PAGE.

The third complex, gcIII, contains two glycoproteins, gp86 and gp145 kDa, gp86 being a homologue of HSV I gH (Cranage et al, 1988). The gene that encodes gp145 has not yet been identified.

1.3.3.2 Nonstructural Proteins

The original classification of proteins into 'immediate early', 'early' and 'late' proteins (Stinski et al, 1980) followed the cascade regulation of protein synthesis found in HSV I infected cells (Honess & Rolzmann, 1974). Immediate early and early proteins were defined as proteins made prior to DNA synthesis, and most appear to be non-structural. As the first proteins to be translated from the viral DNA, the IE proteins must play an important role in establishing infection. Some have been found to be phosphorylated DNA-binding proteins, which cause alteration in cell chromatin structure and template activity (Kamata et al, 1978, 1979). The number of proteins varies from one to three abundant, and four to seven minor proteins (Landini & Michelson, 1988). Some IE proteins are expressed only transiently (Law et al, 1985), whilst at least one persists throughout the replicative cycle (Michelson et al, 1979). Using a number of techniques, workers have described 25 early proteins (Stinski, 1978; Rodgers et al, 1985),
including many DNA binding and nonstructural proteins. The early proteins include phosphoproteins (Michelson et al., 1984), glycoproteins (Stinski, 1977), and enzymes (Huang, 1986), and many have defined or potential regulatory functions.

1.3.3.3 Regulatory Proteins

Four categories of proteins with either direct or indirect regulatory functions have been described (Landini & Michelson, 1988). The first category comprises enzymes either known to be coded by the virus or that are part of the virus particle. After infection, a virus-specific α-type DNA polymerase appears in the cell (Hirai & Watanabe, 1976; Huang, 1986) and has different biochemical properties from the cellular polymerase. A further DNA polymerase, virion-associated cellular α-polymerase, is found within virions (Mar et al., 1981). The essential difference between the two polymerases, virus-specific and virion-associated, is their sensitivity to PAA. The virus-specific polymerase also has a 3'-5'-exonuclease activity which may have a proof reading function (Nishiyama et al., 1983). Virus related protein kinases have been reported by four groups (Landini & Michelson, 1988). The function of the kinases seems to be phosphorylation of viral structural proteins, rather than exogenous protein substrates (Mar et al., 1981). These findings were supported by the work of Roby & Gibson (1986), who found that the kinase activity was similar in specificity and amount of activity for all strains of HCMV studied. Interestingly, they also detected the presence of the kinases in all three HCMV particles, and found that kinase activity correlated with the presence of envelope.
The second category of regulatory proteins comprises cellular enzymes whose activities are stimulated during infection. After infection, there is a generalised stimulation of host cell metabolism (Garnett, 1979; Kanata et al, 1978) and, specifically, of a number of enzymes associated with DNA synthesis (Furlini et al, 1984; Estes & Huang, 1977). The stimulation of these host cell enzymes prior to viral DNA synthesis may be an important factor in successful viral replication.

The third category consists of virus specified DNA-binding proteins (DB). The precise function of these nonstructural proteins is unclear, but they are a diverse group including both IE and E proteins (Musiani et al, 1979). A number of these proteins, however are highly immunogenic in man, in particular HCMV nuclear antigen (CMNA), an 80 kDa protein (Gergely et al, 1980) and immediate early antigen (IEA), a 72-76 kDa protein (Gibson, 1983).

The fourth category comprises phosphoproteins. Phosphorylation or dephosphorylation can have a major effect on both the structure and function of a protein. There is, therefore, the potential for the viral phosphoproteins to regulate activities in the replication cycle (Landini & Michelson, 1988). Many HCMV structural proteins are phosphorylated (Gibson, 1983, Roby & Gibson, 1986), as are a number of important nonstructural proteins (Gibson, 1981; Gergely et al, 1980), of which the IE proteins are already known to play a role in modifying chromatin structure and template activity (Kamata et al, 1978, 1979; Musiani et al, 1981).

1.3.4 Latency

Apart from morphology and certain aspects of the replication cycle, the main feature shared by the herpesvirus group is their ability to
establish latent infection. Latent infection is considered to be a 
persistent infection in which the viral genome is present, and, while 
limited expression may occur, infectious virus is not produced, except 
during episodes of reactivation (Stevens, 1989).

Whilst latency is an essential part of the natural history of herpes 
viruses, the basic mechanisms of establishment and maintenance of the 
latent state and of reactivation are only now being understood in any 
detail. Although there is a great deal of evidence for latency of HCMV, 
most of this is somewhat circumstantial and indirect. The persistence 
of HCMV in a latent state has been well documented by many workers, 
and includes instances where infection is transferred via organ 
transplant from HCMV-Ab positive individuals in whom infectious virus 
cannot be detected at the time of transplantation (Chou, 1986, 1987b) 
to HCMV-Ab negative individuals, transmission by transfusion of HCMV-
Ab positive blood to HCMV-Ab negative individuals (Yeager, 1974), the 
finding that therapeutic immunosuppression leads to active virus 
excretion in most HCMV-Ab positive individuals (Glenn, 1981), and the 
finding of Huang et al (1980) that the restriction patterns of 
repetitive urine and genital tract isolates from a group of women, over 
a period of up to six years, were identical.

Identification of the actual sites of latency, however, has been one of 
the major problems in establishing proof of latency. In patients 
reactivating virus after immunosuppression, virus can readily be 
isolated from urine and saliva, suggesting that the kidney tubules and 
salivary glands may be sites of latency. There is, however, no direct 
evidence for this. Evidence, albeit limited, does exist, however, to 
implicate peripheral blood leucocytes (PBMC) and vascular tissue as 
sites of latency. Two groups of workers (Einhorn & Öst, 1984; Rice et
al, 1984), have shown that a small percentage of normal lymphocytes (2–3%) could be infected by HCMV in vitro, as judged by expression of IE and early proteins, though infectious virus was not produced.

More conclusive evidence was reported by Schrier et al (1985) who detected HCMV IE RNA in PBMC of 8 of 8 HCMV-Ab positive and 1 of 12 HCMV-Ab negative healthy individuals, and by Nelson et al (1990) who detected IE RNA in the peripheral blood leucocytes of 13 of 14 HCMV-Ab positive individuals. The proportion of cells expressing RNA varied from 0·035–2% in the HCMV-Ab positive group and was 0·1% in the one seronegative individual (Schrier et al, 1985), although Nelson et al (1990) found just 0·01% of cells expressing RNA. Recently Stanier et al (1989) demonstrated the presence of HCMV DNA in PBMC of healthy blood donors by the use of the polymerase chain reaction (PCR). They found that PBMC from all of 20 HCMV-Ab positive and 4 of 10 HCMV-Ab negative donors contained viral DNA.

In 1987, Petrie et al (1987), reported finding HCMV DNA in the epithelial tissue from arterial walls using in situ hybridisation with biotin labelled probes. These findings were confirmed by Hendrix et al (1990) who used DNA dot blot, in situ hybridization and PCR to detect HCMV DNA in the smooth muscle in the arterial walls. Both studies linked atherosclerosis with HCMV infection; localised infection in the arterial wall causing vascular damage which contributed to the development of the atherosclerotic lesions.

Although firm evidence to support the latency of HCMV appears to be slowly building, further work is required, especially with respect to the physical state of the viral DNA and the extent of viral transcription during the latent state.
1.4 IMMUNOLOGY OF HCMV INFECTION

Studies in animal models and in humans have shown that the immune response to HCMV infection is very complex. This complexity is attributable to the virus being able to infect cells of the immune system, being capable of stimulating or suppressing the host immune response, and being capable of avoiding host mechanisms for controlling virus replication.

1.4.1 Humoral Immune Response

The humoral immune response to HCMV is generally strong, eliciting production in most infected individuals of Ab of a reasonable titre, which seems to be maintained throughout life without any significant drop. However, the effectiveness of the Ab in protection against HCMV is uncertain as numerous examples of subsequent infection of HCMV-Ab positive individuals have been documented (Grundy et al, 1986; de Cates et al, 1988). However, some studies have shown that although the presence of HCMV-Ab may not prevent infection, it may ameliorate the disease process (Stagno et al, 1982; Glenn, 1981).

It is probable that persistent replication of virus following a primary infection or reactivation of latent virus is associated with a deficiency in cell mediated immunity, irrespective of the presence of Ab; therefore, HCMV-specific cellular immunity has a significant role in recovery from infection. The presence of circulating HCMV-Ab is primarily a marker of previous infection rather than an indication of immune status and, because of the latency associated with HCMV, may also be considered a marker of potential infectivity.
Neutralising HCMV-Ab's have been identified (Waner et al, 1977; Tamura et al, 1980; Pass et al, 1983), often in the presence of free virus. Tamura et al (1980) demonstrated neutralising antibodies in the saliva of 75% of infants despite the presence of virus. However in vivo neutralising activity has not yet been demonstrated and HCMV immune complexes have been found to be capable of infecting cells in culture (Rundell & Betts, 1981). A possible explanation for the ineffective in vivo neutralisation of HCMV is that the viral antigens are masked by a host derived protein, for example by $\beta_2$-microglobulin (McKeating et al, 1986).

1.4.1.1 Isotype Response

The humoral immune response is characterised by the production of IgM, IgG, IgA and IgE Ab to HCMV, of which IgM and IgG have been studied in the most detail. These studies have shown that primary infections usually produce a specific, transient IgM response lasting up to six months (Griffiths, 1981; Kangro et al, 1982) with the subsequent production of persistent HCMV-IgG. Diagnosis of primary infection has therefore been made on the basis of the presence of HCMV-IgM. The presence of HCMV-IgM has also been reported in recurrent infections although the significance is not clear (Kangro et al, 1982; Stagno et al, 1985; Chou et al, 1987a).

The production of HCMV-Ab of other isotypes, namely IgA (Torfason et al, 1981; Sarov et al, 1984; Doerr et al, 1987; van Loon et al, 1987), and IgE (van Loon et al, 1987) has also been reported. HCMV-IgA has been found in a high proportion of infections. Doerr et al (1987) and van Loon et al (1987) both found between 88 and 95% of primary infections marked by an IgA response, although these authors
disagree over the incidence in recurrent infections, finding 100% and 58% respectively. However, most authors agree that HCMV-IgA may be a more useful marker of recurrent infection than HCMV-IgM.

van Loon et al (1987) found a similar transient response for HCMV-IgE. A high incidence of HCMV-IgE (95.8%) was found in primary infections, whereas only 1.5% of healthy seropositive individuals and 7.6% of patients with recurrent infection were found to possess detectable HCMV-IgE.

1.4.2 Cell Mediated Immunity

Cellular immunity to HCMV is an important aspect of the host immune response to the virus, a major factor being that patients with impaired cell mediated immunity are at risk from HCMV disease. Furthermore, reactivation of latent virus at times of impaired immunity may imply that cellular immunity plays an important role in the prevention of secondary infections.

During the acute phase of primary infection with HCMV, cell mediated immunity is depressed, recovering slowly during the convalescent phase. In vitro lymphocyte proliferation in response both to free viral antigen (Ten Napel & The, 1980; Roenhorst et al, 1988) and to cell associated antigens (Roenhorst et al, 1988) is depressed, as are responses to mitogens, allogeneic lymphocytes and recall-antigens. In addition to the HCMV response, only the responses to PHA and allogenic lymphocytes return to normal, the remainder stay depressed, at least during the convalescent phase, up to a year post infection (Roenhorst et al 1988).
During recovery from primary infection both HLA class I and II restricted cytotoxic T lymphocytes (CTL) are produced and these play an important role in containing virus replication (Borysiewicz et al., 1986). Virus-specific killing is mediated by CD8\_CTL and can be inhibited by HLA class I monoclonal Ab (Laubscher et al., 1988). These authors also found that most of the earlier\_CTL produced were directed against the IE antigens and, therefore, these\_CTL may be important in restricting virus replication by eliminating the infected cells at an early stage of infection. They further demonstrated lysis by CD4\_CTL. These\_CTL are HLA class II restricted, although class II monoclonal antibodies did not inhibit cell killing, possibly due to NK cell activity. The role of\_CTL in HCMV infections is probably critical as the ability to develop a specific cytotoxic response is correlated with successful resolution of disease (Meyers, 1984; Quinnan, 1982).

Abnormalities in CMI in infants and children with congenital and perinatal infections have been found to differ from those in adults with HCMV disease, and suppression of CMI lasts much longer than in adults. The effect consistently seen in infants was impairment of the lymphocyte proliferation response (LPR), although Ab was produced normally (Gehrz et al., 1977; Starr et al., 1979). Cauda et al. (1987) subsequently found that in symptomatic infants NK cell activity was also impaired.

HCMV infection causes long-lasting and profound disturbances in the host cellular immune response, possibly due to the persistence of the virus in the latent state. Cellular immunity to cell associated HCMV antigens develops gradually after primary infection, persists for many years and is vital for maintaining the latent state.
1.4.3 Other Mechanisms of Host Immune Response

1.4.3.1 Neopterin

Neopterin is a low molecular weight compound derived from GTP and produced by macrophages after stimulation by IFN-γ (Fuchs et al., 1988). Neopterin levels are high during acute HCMV infection and fall slowly as HCMV-specific antibodies appear. Production is closely correlated with activation of cell mediated immunity.

1.4.3.2 Natural Killer Cells

The role of natural killer (NK) cells in immunity to HCMV infection is still unclear, although they are probably important in primary infection as a first line of defence prior to Ab and Tc cell production. NK cells have been shown preferentially to lyse HCMV infected cells in vitro (Starr & Garrabrants 1981, Borysiewicz et al. 1985, Bandyopadhyay et al. 1988), lysis being dependent upon expression of HCMV-EA (Borysiewicz et al., 1985). NK lysis of uninfected cells led Borysiewicz et al. (1985) to suggest that the NK cells recognised a receptor present on all cells, but increased in expression on infected cells.

1.4.3.3 Interferon

Interferon production is a further aspect of CMI that is affected by HCMV infection. The interferon response of mononuclear blood cells from patients with HCMV mononucleosis stimulated by HCMV antigen (Levin et al., 1979) or mitogens (Rinaldo et al., 1980) was found to be diminished up to fourfold compared to cells from normal healthy individuals, the response returning to normal during convalescence. The response of other cell types involved in CMI is, however, somewhat different. NK cell activity may be enhanced by activation of
the cells by the IFN-α pathway. HLA-DR+ accessory cells are required for lysis by NK cells. These cells produce IFN-α, which then stimulates the NK cells to mediate lysis of target cells (Bandyopadhyay et al., 1986; Bandyopadhyay et al., 1988). Virus specific killing is enhanced dramatically by pretreatment of infected cells with IFN-γ (Laubscher et al., 1988). The mode of action may involve the expression of HLA I on cell surfaces as Tc activity is HLA class I restricted, and IFN-γ increases the amount of HLA class I antigens expressed and stimulates expression of HLA class II antigens (Pober et al., 1983; Bukowski & Welsh, 1985).

1.4.3.4 Macrophages

Macrophages are an important component of the immune response to viral infections, although only limited information on the mechanisms of this activity is available (Skamene & Gros, 1983). Co-cultivation of monocyte derived macrophages (MdM) with HCMV-infected fibroblasts was found to inhibit infection of the fibroblasts; the inhibitory effect being dependent on the virus moi, showing greater inhibition at a lower moi (Manor & Sarov, 1988).

1.4.4 Evasion of Host Defence Mechanisms

Evasion of, or interference with, the host defence mechanisms may assist the process of primary infection and, importantly, the induction of the latent state. HCMV appears to have developed two mechanisms by which it can evade, to some extent, the host immune response, facilitating infection and subsequent transmission of the virus.
1.4.4.1 \( \beta_2 \)-Microglobulin

The inability of an EIA to detect HCMV excreted in urine led McKeating et al. (1986) to propose that the virus was effectively masked by a coating of low molecular weight host protein. This protein was shown to be \( \beta_2 \)-microglobulin (\( \beta_2m \)), an 11.7 kDa protein which is synthesised by all somatic cells and forms the light chain moiety of class I HLA molecules. Binding of \( \beta_2m \) to the 45 kDa heavy chain forms the complete molecule (Rebai & Malissen, 1983). Grundy et al. (1987a) concluded from *in vitro* studies that \( \beta_2m \) was bound after release of the virus from cells, and predicted that excreted HCMV found in all body fluids would be coated with host \( \beta_2m \). McKeating et al. (1987) showed that this was indeed the case and that \( \beta_2m \) was bound to the envelope of HCMV. They further surmised that the coating of the virus enabled it to avoid the host immune response and thus facilitated its transmission.

Enhancement of the infectivity of HCMV due to binding of \( \beta_2m \) has been shown by Grundy et al. (1987b) who proposed that HCMV may exploit the bound \( \beta_2m \) to bind to host cells, and have shown that \( \beta_2m \) and HCMV compete for the same binding site on host cells. They have further found that coated virus can use class I HLA molecules on the cell surface as receptors. *In vitro* exchange of \( \beta_2m \) between cell surface and medium has been reported, favouring displacement of the surface molecule (Sanderson et al., 1985). It is thus probable that the \( \beta_2m \) bound to HCMV would displace the surface \( \beta_2m \) and hence facilitate virus binding and cell infection. Recently, Stannard (1989) has used immunogold labelling to study \( \beta_2m \) binding to HCMV. The findings were in general agreement with the previous studies, except that \( \beta_2m \) was found to bind to the tegument of the virus rather than the envelope.
It was postulated that the tegument proteins may play an important role in the binding and infectivity of damaged or incomplete virions.

1.4.4.2 Fc Receptor Production

Cells infected with HCMV develop Fc receptors on their surface (Furukawa et al, 1975b; Westmoreland & Watkins, 1974). This receptor is a glycoprotein of 42 kDa (Sakuma et al, 1977) and appears from around 36 hours post infection. The action of the receptor is to bind IgG non-specifically and this appears to prevent immune recognition of the infected cell by acting as a blocking Ab, affording the cell considerable protection against Ab or cell mediated immune lysis (Adler et al, 1978).

1.5 DETECTION OF HCMV INFECTION

1.5.1 Detection of Virus

Diagnosis of an active HCMV infection cannot be made solely on the basis of clinical symptoms: these may range from asymptomatic to overt mononucleosis with severe organ damage or may resemble the symptoms that may be found in diseases caused by a number of other infectious agents. Diagnosis of HCMV infection can be achieved by detection of specific, established markers; such as distinctive nuclear and cytoplasmic inclusions in cytomegalic cells (Shulman et al, 1982), rising titre of circulating HCMV-Ab (Panjwani et al, 1985), isolation of virus in cell culture (Gleaves et al, 1985), immunohistochemical detection of HCMV proteins (Hackman et al, 1985), or detection of HCMV DNA by in situ hybridisation (Niedobitek et al, 1988), dot-blot
hybridisation (Augustin et al, 1987), or, more recently, the polymerase chain reaction (PCR) (Olive et al, 1989a).

1.5.1.1 Culture of Virus in vitro

Isolation of virus by culture is performed on diploid human fibroblast monolayers prepared from embryonic tissue. After infection with virus, the cells may show a cytopathic effect (cpe) any time between one day and several weeks post-infection depending upon the moi of the virus, although growth of the virus is usually slow, taking 2 to 3 weeks, or even longer with some isolates, to reach 80 to 100% cpe. The cpe, which is characteristic and may need no confirmation, appears as foci of enlarged, rounded, refractile cells which spread through the monolayer following the pattern of the uninfected cells, with eventual detachment of the cells. Most workers consider that culture for at least four weeks is necessary to allow production of cpe from low titre, low infectivity isolates, and only after this period may the culture be deemed to be negative.

A number of different sources of potential isolates may be examined by cell culture, although possible toxicity of the sample to the cells must be considered. Probably the most commonly examined specimen is urine (Gleaves et al, 1985; Janssen et al, 1988; Leland et al, 1989) which is also the best source of virus in most patient groups at risk of HCMV infection; though bronchoalveolar lavage fluid (BAL) (Stirk & Griffiths, 1988; Crawford et al, 1988), buffycoats (Schirm et al, 1987; Steel et al, 1988), and throat swabs and saliva (Rush et al, 1989; Woods & Thiele, 1989), may also be tested using conventional cell culture techniques.
Because of the long incubation times required for detection of virus, more rapid techniques have evolved which are based on culture, but do not require development of cpe. Gleaves et al (1984) reported the use of a technique involving the centrifugation of virus from samples onto cell monolayers on circular coverslips in sealed vials - shell vial culture. This technique involves the use of monoclonal antibodies to detect HCMV specific proteins after a short incubation period (16 to 36 hours) and is more sensitive than conventional culture (Gleaves et al., 1985; deGirolami et al., 1987). Lipson et al. (1990) found that better results could be obtained from urine samples using shell vial culture if they were clarified prior to inoculation. MacKenzie & McLaren (1989) found that the sensitivity of the technique could be increased by using mink lung cells rather than human fibroblasts.

1.5.1.2 Use Of Monoclonal Antibodies (MoAbs)

The availability of MoAbs has had a dramatic impact on the detection of viral antigens in cultured cells. These can be used in conjunction with a variety of techniques, including in situ hybridisation, detection of virus in cultured cells, and antigen capture assays and are usually visualised by conjugating the Ab with a fluorescent dye or with an enzyme followed by a chromogenic substrate.

The most common use has been to detect IEA production in cultured cells, as in the shell vial technique described above (Gleaves et al., 1984), providing a rapid diagnosis of infection. Griffiths et al. (1984) used MoAbs to detect early antigen production in slide cultures by immunofluorescence, 'detection of early antigen fluorescent foci (DEAFF)'. This test gave results after 27 hours compared with an average of 17.5 days by conventional culture, and had 100% specificity.
and 80% sensitivity. A further report on the same technique involving a larger sample number showed 94% agreement between culture and the DEAFF test, with equal numbers of samples (2.8%) giving discrepant results by either technique. The same principle has been applied by other workers, using immunoenzymatic staining rather than immunofluorescence (Swenson & Kaplan, 1985; Steel et al, 1988; Schacherer et al, 1988).

MoAbs can also be used for the detection of HCMV antigen in representative tissue sections using in situ techniques, enabling the spread of the virus to be followed in various organs and providing important information concerning the pathogenesis of HCMV. Infections in many tissues and from various patient groups have been studied using this method (Goldstein et al, 1982; Porter et al, 1990; Jiwa et al, 1989; van der Bij et al, 1988). Importantly, HCMV antigens have been demonstrated in morphologically normal tissue from patients with confirmed infection (Jiwa et al, 1989), and from healthy HCMV-Ab positive individuals (Toorkey & Carrigan, 1989). Very often, the combined use of MoAbs and DNA hybridisation in situ enables detection of active infections (Porter et al, 1990).

Antigen capture assays have been developed; these apply immobilised MoAbs (McKeating et al, 1985) or polyclonal antibodies (El-Mekki et al, 1987) to 'capture' HCMV virions, which may then be detected using a second HCMV specific, enzyme-conjugated Ab. The value of such an assay is that virus culture is not required, the assay is rapid, and it has the potential for standardisation and therefore large scale commercial production. Unfortunately, the results of studies using such assays have not been as expected. McKeating et al (1985) detected HCMV in 65% of culture positive urines, but only after prior
storage at 4°C, and El-Mekki et al. (1987) found that repeated freeze/thaw cycles increased the sensitivity of their assay.

Although MoAbs have proved to be very useful for the detection of virus and virus infected tissue in histological specimens, problems may arise due to their high specificity. In situations where antigenic variation exists, a particular epitope may not be recognised by a MoAb specific for another HCMV strain. Chou & Scott (1988) reported such a situation, where two of 21 strains of HCMV were not recognised by a MoAb against the major IE viral protein.

1.5.2 DNA Hybridisation

The detection of viral DNA or RNA in clinical specimens can be achieved by hybridisation with specific nucleic acid probes. The technique may allow either direct detection of nucleic acid in sections of infected tissue - *in situ* hybridisation, or detection of extracted, partially purified nucleic acid immobilised on a solid support - dot or slot blot hybridisation. Hybridisation may be demonstrated by a number of different methods depending on the choice of label for the probe; radioactive, biotinylated, enzymic, fluorescent or antigenic labels. Radioactivity has been, and continues to be, the most commonly used label, but the use of biotin is increasing, especially in diagnostic applications, as it offers a safe alternative to radioisotopes and is stable over long periods.

1.5.2.1 Dot/Slot Blot Hybridisation

Dot-slot blot hybridisation, using radioactive or biotin labelled probes, has frequently been used for the detection of HCMV in urine samples, especially in situations where large numbers of samples are involved.
(Chou & Merigan, 1983; Virtanen et al, 1984; Schuster et al, 1986; Buffone et al, 1988). When compared with cell culture, the sensitivity of these assays ranged from 70-93%, with the predictive value of a negative result ranging from 80-97%. In a prospective study involving 67 buffycoat samples from bone marrow transplant patients, Spector et al (1984) detected HCMV DNA by dot blot hybridisation in 13 of 14 (93%) culture positive samples, and in 21 of 53 culture negative samples. In 20 of 21 of these samples evidence was presented that the hybridisation results were correct and that the technique was more sensitive than the co-cultivation method used to detect HCMV. An important feature of dot/slot blot hybridisation is that virtually any clinical specimen can be used; intact tissue sections are not required, and importantly, time consuming and expensive cell culture is not required.

1.5.2.2 *In Situ* Hybridisation

Evidence for active or silent HCMV infection in specific tissues can be obtained by the use of *in situ* hybridisation. Currently, most workers use biotin labelled probes and enzymatic detection of the hybrids. Using *in situ* hybridisation it is possible to detect HCMV DNA and RNA in tissues with and without morphological evidence of infection. The combined use of *in situ* hybridisation and immunohistochemistry using MoAbs can provide accurate information concerning the state of the infection in the tissues.

*In situ* hybridisation has been used to detect HCMV infection in a variety of tissues. HCMV infection may underlie another infection or disease state, for example Keh & Gerber (1988) used *in situ* hybridisation and immunohistochemistry to investigate HCMV infection
in AIDS patients. In addition to finding HCMV DNA and viral antigens in cytomegalic cells, they also found both in morphologically normal cells, which account for 25-55% of infected cells in systemic HCMV infection in AIDS patients. Further studies have demonstrated the presence of HCMV in colon biopsy samples (Roberts et al, 1988; Clayton et al, 1989), and in bronchoalveolar lavage fluid from AIDS and other immunosuppressed patients (Hilborne et al, 1987; Jiwa et al, 1990), in liver biopsies from patients undergoing liver transplant (Naoumov et al, 1988; Dény et al, 1989), and in PBMC from immunosuppressed patients (Dankner et al, 1990).

1.5.3 Polymerase Chain Reaction

Although the theoretical basis for PCR has been understood for some time, the procedure was previously very laborious and relatively inefficient. Now it is recognised as a major advance in practical molecular biology. The two factors that have transformed the technique are the production of automated temperature cycling equipment and the isolation of thermostable DNA polymerases (eg Taq DNA polymerase). The two factors together mean that the reaction mix can be prepared with sufficient enzyme for the whole procedure, and that the automated temperature cycler will perform the procedure without any manual involvement.

PCR has revolutionised the detection of infectious agents, especially those present in small numbers and those that integrate into the genomic DNA. The technique has proved especially useful for the detection of virus infections, and has been reported to be capable of detecting a single copy of viral genome in as many as $10^5$ cells (Saiki
et al, 1988), a level of sensitivity previously only approached by lengthy \textit{in situ} hybridisation techniques.

A number of studies have been published detailing the use of PCR for the detection of HCMV in clinical samples. Using oligonucleotide primers specific for the immediate early (IE) region of the HCMV genome, clinical samples from congenitally infected infants (Demmler \textit{et al}, 1988), bone marrow transplants (Cassol \textit{et al}, 1989; Jiwa \textit{et al}, 1990) renal transplants (Olive \textit{et al}, 1989a, 1989b) and AIDS patients (Shibata \textit{et al}, 1988; Shibata & Klatt, 1989; Hsia \textit{et al}, 1989) have been examined by PCR in parallel with standard techniques. The technique is at least as sensitive as virus culture, and some authors (Hsia \textit{et al}, 1989; Olive \textit{et al}, 1989b; Shibata \textit{et al}, 1989) have reported greater sensitivity together with a significant decrease in the time taken to confirm infection. PCR has recently been used to demonstrate latent HCMV DNA in PBMC from healthy HCMV-Ab positive individuals (Stanier \textit{et al}, 1989; Morris \textit{et al}, 1989).

1.5.4 Detection of HCMV-Specific Antibody

The advent of methods enabling the rapid detection of acute infections, such as monoclonal antibodies and DNA probes, have to a certain extent replaced conventional serology, although this is still invaluable in certain instances. Knowledge of the serological status of organ donors is important since the transplanted organ itself is known to be an important source of infection (Grundy \textit{et al}, 1988). The serological status of the recipient is also important as the incidence and severity of HCMV disease is higher in HCMV-Ab negative recipients (Smiley \textit{et al}, 1985). Most cases of maternal HCMV infection during pregnancy and subsequent congenital infection are initially
diagnosed solely on the basis of the presence of HCMV-Ab appearing in an otherwise healthy individual (Stagno et al, 1986).

Enzyme immunoassay (EIA) is the most sensitive test routinely used to detect circulating Ab, whilst tests such as complement fixation (CF) and latex particle agglutination are still routinely used in a number of laboratories. Immuno (Western) blotting (IB), has some application in the detailed analysis of the response to individual HCMV proteins and the isotypic response of the infected individual.

Rapid diagnosis of HCMV infection in transplant patients is essential to allow differentiation between HCMV infection and graft rejection, and thus the correct treatment regimen. Miller et al (1989) compared the time taken for seroconversion with detection of viruria in a group of renal allograft recipients. Although the method of Ab detection had some bearing on the point of detection of seroconversion, this was generally prior to the detection of viruria by conventional culture.

1.5.4.1 Detection of HCMV-IgM Antibody

Diagnostic Ab screening involves detection of circulating HCMV-IgM and IgG antibodies, and interpretation depends upon the generally accepted precept that IgM is produced immediately following infection and then declines as IgG appears. HCMV-IgM to late antigen (LA) is produced after primary infection. It usually persists for 3 to 4 months and is therefore associated with recent or current infection. However, HCMV-IgM has occasionally been found to persist for longer periods, even up to 2 years (Pass et al, 1983; Sutherland & Briggs, 1983). Transient HCMV-IgM has also been demonstrated in 6 of 21 (Kangro et al, 1982) and 19 of 38 (O'Neill et al, 1988) transplant patients undergoing recurrent infection. The Ab had a lower titre than that
produced in primary infection and was produced within 2 to 3 months of infection.

Complement fixing activity has been associated with IgM Ab produced in response to infection by a small number of viruses including HCMV (Booth & Mohammed, 1988). It is thought that this activity helps to eliminate infected cells and is linked with the expression of the HCMV membrane antigens (Middeldorp et al, 1986).

Many workers have used either RIA or EIA to detect circulating HCMV-IgM Ab (Kangro, 1980; Stagno et al, 1985; van der Giessen, 1990), although fluorescent techniques have been used by others (Elder & Smith, 1987; Zerbini et al, 1986; Thomson et al, 1987). Major factors leading to the loss of specificity associated with screening for IgM antibodies, are the non-specific binding of rheumatoid factor (IgM antibodies to IgG) (Fung & Tilton, 1985; Salonen et al, 1980), and the presence of high levels of specific IgG (Buimovici-Klein et al, 1983).

1.5.4.2 Detection of HCMV-IgG Antibody

In individuals with primary HCMV infection, circulating HCMV-IgG Ab is produced late after infection, when symptoms have appeared. The Ab generally persists for life, although fluctuations in titre have been reported. Following recurrent infection, a significant increase in titre of Ab is invariably found (Pass et al, 1983; O'Neill et al, 1988). O'Neill et al (1988) further examined the specific response to HCMV-EA and HCMV-LA. They found that, following primary infection, IgG-EA appears later than IgG-LA, but that after recurrent infection the titre of both antibodies increased in parallel. Significant rises in titre of IgG-EA were found in all patients with recurrent infection but in only 40% of patients with primary infection. The value of IgG-EA in the diagnosis
of recurrent infection is limited, however, as it has been found both in pregnant women (Griffiths et al, 1980) and in normal, healthy seropositive individuals (Friedman et al, 1982).

RIA and EIA are the assays of choice for the detection of HCMV-IgG as they offer the greatest sensitivity and specificity, together with ease of use and the ability to handle of large numbers of samples. A number of other techniques are also used, including complement fixation (CF) immunofluoresence (IF), and latex particle agglutination, of which latex particle agglutination has the advantage of being simple, rapid, and relatively sensitive (Chou & Scott, 1988; Klinedinst et al, 1988), for example for the rapid screening of allograft donors and recipients (Leland et al, 1989).

1.6 HCMV AND BLOOD TRANSFUSION

There can be no doubt that, in the past, blood transfusion represented a major source of HCMV infection in those patient groups known to be at risk. Today, however, whilst the potential for transmission of HCMV has not changed, the incidence of transmission has dramatically decreased as an awareness of the problem and approaches to its solution have evolved.

1.6.1 Early Findings

The first evidence for transmission of HCMV by transfusion was reported by Kreeel et al (1960), who described a febrile mononucleosis three to eight weeks after open heart surgery involving blood transfusion, the post perfusion syndrome. The identification of HCMV as the specific cause of this syndrome was made by Klemola et al
(1965) and Kaariainien et al. (1966a), who reported rises in titre of CF antibodies to HCMV, mild mononucleosis, and, in one case (Kaariainien et al., 1966b), isolation of HCMV from the urine of a patient. Both Stulberg et al. (1966) and Harnden et al. (1967) isolated HCMV from peripheral blood, supporting the hypothesis of potential transmission of HCMV by blood transfusion. Lang et al. (1968) described four cases of HCMV mononucleosis in a group of 131 patients who had undergone open heart surgery. Virus was recovered from the urine of all four and from the throat or blood of three. Lang & Hanshaw (1969) described four symptomatic cases of HCMV infection where virus was cultured from the buffycoats, and HCMV-IgM subsequently detected in all four.

Subsequently, a large number of prospective studies have been performed and these have provided information concerning the clinical and laboratory diagnosis of infection in patients and, more importantly, have considered the relative risks of post-transfusion HCMV infection, and factors mediating these risks. Table 1.1 summarises the results of twelve such studies performed between 1966 and 1988 and which include more than 50 patients.

The incidence of infection varies from study to study with an overall figure of 19%. Interestingly, the overall value from each individual study appears to decrease as the studies become more recent, a finding that correlates with a gradual decrease in the average number of units transfused per patient. The incidence of symptomatic infection was higher in primary (9.2%) than in secondary infections (4%). The overall incidence of symptomatic infection post-transfusion was 2.0%, of which 1.7% was attributable to primary and 0.3% to secondary infection. However, these figures are based on a definition of secondary infection based upon a four fold rise in HCMV-Ab titre.
post-transfusion, a definition which may lead to overestimation because of passively acquired high titre HCMV-Ab.

Table 1.1 Incidence of primary and secondary HCMV infection after transfusion

<table>
<thead>
<tr>
<th>Reference</th>
<th>Total pts</th>
<th>Seronegative recipients</th>
<th>Seropositive recipients</th>
<th>Mean no. Symptomatic rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Txed Inf (%)</td>
<td>Txed Inf (%)</td>
<td>Txed Inf (%)</td>
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<tr>
<td>Paloheimo et al 1968</td>
<td>63</td>
<td>19 (30)</td>
<td>17 10 (59)</td>
<td>46 9 (20)</td>
</tr>
<tr>
<td>Henle et al 1970</td>
<td>152</td>
<td>53 (35)</td>
<td>61 36 (59)</td>
<td>91 17 (19)</td>
</tr>
<tr>
<td>Prince et al 1971</td>
<td>152</td>
<td>30 (20)</td>
<td>93 17 (18)</td>
<td>59 13 (22)</td>
</tr>
<tr>
<td>Caul et al 1971</td>
<td>55</td>
<td>21 (38)</td>
<td>21 7 (33)</td>
<td>34 14 (41)</td>
</tr>
<tr>
<td>Luthardt et al 1971</td>
<td>73</td>
<td>12 (16)</td>
<td>35 8 (23)</td>
<td>38 4 (11)</td>
</tr>
<tr>
<td>HRC Work. Party 1974</td>
<td>712</td>
<td>37 (5)</td>
<td>270 24 (9)</td>
<td>442 13 (3)</td>
</tr>
<tr>
<td>Monif et al 1976</td>
<td>207</td>
<td>19 (9)</td>
<td>140 16 (11)</td>
<td>67 3 (4)</td>
</tr>
<tr>
<td>Armstrong et al 1976</td>
<td>119</td>
<td>15 (13)</td>
<td>93 9 (10)</td>
<td>26 6 (23)</td>
</tr>
<tr>
<td>Bayer 1977</td>
<td>159</td>
<td>5 (3)</td>
<td>30 1 (3)</td>
<td>129 4 (3)</td>
</tr>
<tr>
<td>Alter et al 1982</td>
<td>107</td>
<td>22 (21)</td>
<td>29 14 (48)</td>
<td>78 8 (10)</td>
</tr>
<tr>
<td>Wilhelm et al 1986</td>
<td>595</td>
<td>7 1.2</td>
<td>595 7 1.2</td>
<td>- - - 3.4 2 prim 0.35</td>
</tr>
<tr>
<td>Preiksaitis et al 1988a</td>
<td>637</td>
<td>6 (1)</td>
<td>637 6 (1)</td>
<td>- - - 9.2 4 prim 0.1</td>
</tr>
</tbody>
</table>

| Total               | 3031      | 246 (9)                | 2021 155 (7)           | 1010 91 (16)                 |

NG - not given, prim - primary infection
Prince et al (1971) found that only 7% of patients receiving a single unit of blood compared to 21% of patients receiving multiple units demonstrated equivalent rises in HCMV-Ab titre. This finding is in agreement with those of Armstrong et al (1976), Henle et al (1970), Monif et al (1976) and Bayer (1977) who have all reported a higher incidence of infection in multiply-transfused patients than in those receiving less than four units. Early reports linking the age of blood with transmission of HCMV (Kaariainien et al, 1966b; Palohelmo et al, 1968) arose from studies involving open heart surgery, where large volumes of fresh blood were used. Data from transplant recipients were limited at this time as few transplants were performed.

In addition to the serological status of the recipient, the status of the transfused blood was thought to be important in the transmission of HCMV (Luthardt et al, 1971; Monif et al, 1976; Kumar et al, 1980). Luthardt et al (1971) were the first to show the importance of the serological status of the donor blood in that HCMV-Ab negative patients receiving HCMV-Ab negative blood remained uninfected. These findings were corroborated by Monif et al (1976), who demonstrated that the transfusion of HCMV-Ab negative blood to HCMV-Ab negative patients dramatically reduced the incidence of post-transfusion HCMV infection, 3.5% compared to an incidence of 24% when HCMV-Ab positive blood was transfused.

Interestingly, post-transfusion HCMV still occurred, although there are a number of possible explanations for this such as coincidental concomitant infection with exogenous virus or insensitive Ab testing failing to detect HCMV-Ab positive donors with low Ab levels or the seropositivity of recipients.
1.6.2 Carriage of Virus (Latency) in Donor Blood

A candidate vehicle for the carriage of latent virus in blood had been sought for some time when Fiala et al (1975) postulated that PBMC harboured the virus. Subsequently, both Lang et al (1977) and Tolkoff & Rubin (1978) reported that patients who received frozen/recovered red cells had a lower incidence of post-transfusion HCMV than a similar group of patients who received conventional blood. These findings were confirmed by Betts et al (1979), who reported a two year study during which none of 38 HCMV-Ab negative patients who received frozen/recovered red cells developed post-transfusion HCMV infection. These findings led to the conclusion that HCMV was indeed transmitted in the leucocyte fraction of donor blood, and thus transfusion of the normal cellular components of blood such as red cell preparations, platelet concentrates and leucocyte concentrates is associated with a high risk of HCMV infection, whilst the non-cellular components such as fresh plasma, clotting factor concentrates and albumin solutions are essentially free from risk.

During acute infection, HCMV can be detected by DNA hybridisation in all fractions of whole blood and may be isolated from PBMC by culture techniques. However, when these techniques are applied to donor samples virus cannot usually be detected, although inoculated HCMV has been found to persist for up to 28 days in stored whole blood and for up to 97 days in frozen plasma (Rinaldo et al, 1977). Jackson et al (1987) used spot hybridisation to examine the PBMC of HCMV-specific IgM Ab positive blood donors for the presence of HCMV DNA, but without success. There has been only one report of the isolation of infectious virus from donor blood (Diosi et al, 1969), where virus was cultured by normal techniques from the PBMC of two healthy
HCMV-Ab positive donors. This finding has never been repeated and, subsequently blood from more than 1200 HCMV-Ab positive and negative donors has failed to yield any infectious virus (Mirkovic et al, 1971; Kane et al, 1975; Bayer & Tegtmeyer, 1976; Armstrong et al, 1976).

Transmission of HCMV by blood in which no infectious virus can be detected is consistent with the hypothesis that, in healthy individuals who have recovered from previous HCMV infection, latent virus may be harboured in the PBMC. Transfusion of these cells during blood transfusion is the probable means by which the virus is transmitted, although transmission of virus during active infection cannot be ruled out.

Many reports cite transfusion of large amounts of blood from multiple donors, and in these situations the transfusion of a unit of blood from a viraemic, asymptomatic, infected donor is a possibility. However, the finding that 13.5% of multiply transfused neonates develop symptomatic HCMV infection demonstrates an infection rate far higher than would be expected if transmission were due only to the use of blood from acutely infected donors. Thus, the majority of cases of infection of HCMV-Ab negative recipients, and possibly also of HCMV-Ab positive recipients, are due to reactivation of transfused latent virus. Toplin et al (1985), however, reported the transmission of HCMV to two infants who had both received red cells from the same unit of donor blood. In this case, virus was cultured from the urine of the donor one year after the implicated donation; the donor had HCMV-IgG and virus could not be cultured from any other source. The virus isolated from the donor was compared by restriction enzyme analysis to isolates from the two infected infants and found to be identical.
This is the first and only report of transfusion transmission confirmed by molecular techniques; it also is highly likely that this donation was made during an acute asymptomatic infection at the viraemic stage.

Molecular evidence for the latency of the virus has been provided recently using *in situ* hybridisation (Schrier *et al*, 1985; Nelson *et al*, 1990) and PCR (Stanier *et al*, 1989) to demonstrate HCMV DNA and RNA in PBMC. The mechanisms of virus reactivation are unclear, but the higher incidence of post-transfusion HCMV infection in immunoincompetent compared to immunocompetent individuals may indicate the involvement of the immune system of the recipient. Reynolds *et al* (1979) postulated that, in infants, the immaturity of the immune system meant that they could not prevent reactivation of the virus or neutralise reactivated virus. Lang (1972) proposed that transfused PBMC may trigger an allograft reaction leading to reactivation of virus latent in the recipient. Cheung & Lang (1977) further proposed that allogeneic stimulation of the transfused, latently infected PBMC induces reactivation. This theory was based on the observations in mice that transfusion of latently infected blood into allogeneic and syngeneic recipients resulted in more frequent reactivation in the allogeneic recipients.

### 1.6.3 Post-Transfusion HCMV Infections

Post-transfusion HCMV infection may be due to primary infection, reactivation of latent infection or reinfection by free or latent transfused virus. In healthy immunocompetent individuals, post-transfusion HCMV infection is usually asymptomatic, but may manifest as a mild heterophile negative mononucleosis syndrome. There is minimal morbidity associated with such infections and therefore little
need for blood screened negative for HCMV for these patients. Infection in immunoincompetent patients however, may be associated with significant morbidity and mortality. This group of patients includes preterm infants, immunosuppressed infants and children, pregnant women, patients with malignancies undergoing immunosuppressive treatment, AIDS patients, and allograft recipients. Such patients may benefit from transfusion support with HCMV-negative screened blood.

1.6.4 Prevention of Transfusion Transmission of HCMV

Prevention of transfusion associated HCMV infection can be achieved by either removing the leucocytes from the transfused products (Murphy et al, 1988; de Graan-Hentzen et al, 1987) or by the transfusion of HCMV-Ab negative blood to those patients at risk of HCMV infection (Bowden et al, 1987; Tegtmeier, 1988).

1.6.4.1 Use of Leucocyte Poor Blood

Removal of leucocytes from transfused blood can be achieved by a number of methods including freeze/thaw, washing and filtration. The effectiveness of freeze/thaw was reported by Adler et al (1984), who found no cases of post-transfusion HCMV infection in 34 low birth-weight infants transfused with frozen/recovered red cells, compared to infection in 7 of 25 infants transfused with conventional blood. Conflicting reports of the efficacy of 'washed cells' have appeared. Williams et al (1984) reported that the use of washed cells decreased the risk of HCMV infection in neonates, whilst Demmler et al (1985) found no improvement. Washed cells, however, are more variable in their residual leucocyte content; the process is not as efficient as freeze/thawing. Filtration of whole blood has been used for some time
for prevention of alloimmunisation to HLA antigens in multi-transfused patients (MacGregor et al, 1983), and for the removal of leucocytes from platelet concentrates (Sirchia et al, 1983). In a recent study, Gilbert et al (1989) found that 9 of 42 HCMV-Ab negative infants who received unfiltered blood developed post-transfusion HCMV infection compared with 0 of 30 similar infants receiving filtered blood.

1.6.4.2 Screening of Donors for HCMV-Antibody

By far the cheapest and most convenient method of preventing post-transfusion HCMV infection is the screening of donor blood for HCMV-Ab. Whilst the detection of IgG or total Ab is routinely used to determine those donations at risk of transmitting HCMV, a number of studies have investigated the potentially greater infectivity of donors with circulating HCMV-IgM.

1.6.4.3 Screening of Donors for HCMV-IgM Antibody

Presence of HCMV-IgM in primary infections generally indicates current or recent infection and has been reported to mark virus replication (Rasmussen et al, 1982). On this basis, donors possessing circulating HCMV-IgM may be more likely to be viraemic and, therefore, more likely to transmit infection. Tegtmeier (1985) reported the results of unpublished studies which demonstrated a significant correlation between transfusion of HCMV-IgM positive donor units and transmission of virus. The prevalence of HCMV-IgM was found to agree with the calculated donor carrier rates. Beneke et al (1984) found that, although there was correlation between HCMV-IgM and transmission, only half of the infections in their study would have been prevented if IgM positive blood had been excluded, and therefore it was doubtful if HCMV-IgM screening had any value. Wielaard et al
(1986) also concluded that screening for HCMV-IgM alone would not prevent transmission of HCMV. They found that only 0.16% of Dutch blood donors possessed HCMV-IgM, a figure that was considerably lower than the estimated percentage (2.5-12%) of donors capable of transmitting HCMV (Adler, 1983), but in agreement with their calculated local annual seroconversion rate.

1.6.4.4 Screening of Donors for HCMV-IgG Antibody

Routine screening of blood donations involves screening of donor serum samples for the presence of HCMV-IgG. Donors found to be negative by these assays are presumed to present little risk of transmission of HCMV and can be transfused to patients at risk of HCMV infection. All of the published studies investigating the prevalence of HCMV-Ab in blood donors are based on the detection of HCMV-IgG or total Ab.

The prevalence of HCMV-IgG in blood donors varies throughout the world. Krech (1973) reported prevalences in blood donors ranging from 40-79% in developed industrialised countries to 81-100% in third world countries. The prevalence of HCMV-Ab was found to be inversely proportional to the socio-economic status of the population. In developed countries, infection occurred more commonly during adulthood, whilst in third world countries infection occurred very early in life. In the UK, Perham et al (1971) found that 55% of random blood donors had CF Ab to HCMV. More recent UK studies using EIA have found prevalences of 43 to 44% (Hunt et al, 1984; Grint et al, 1985; Eley, 1987 - unpublished data). Interestingly, Klapper et al (1990), have reported the much higher figure of 53% in blood donors from the Manchester area using a new immunoradiometric (RIST) assay.
1.6.5 Effectiveness of Preventative Measures in Transfusion practice.

Yeager et al (1981) provided clear evidence that transfusion was an important source of acquired infection in low birth weight infants. None of 90 infants receiving HCMV-Ab negative blood developed HCMV infection, whereas 10 of 74 infants receiving unscreened blood developed infection. HCMV disease developed in five of these infants of whom four died. These findings were confirmed by other studies (Adler et al, 1984; Lamberson et al, 1983; Smith et al, 1984). Bowden et al (1987) reported that only one of 104 HCMV-Ab negative transplant recipients developed HCMV infection after transfusion of HCMV-Ab negative blood. Mackinnon et al (1988) described similar findings in a group of 37 HCMV-Ab negative BMT recipients.

The current recommendations from the American Association of Blood Banks (AABB) provide for the use of HCMV-Ab negative blood for: HCMV-Ab negative neonates weighing less than 1,250gm, HCMV-Ab negative pregnant women, and bone marrow transplant recipients receiving prophylactic granulocyte transfusions and have been followed since 1984 (Lamberson, 1988). It is recognised that there are other groups of patients who may also require HCMV-Ab negative blood; these may include organ transplant patients, AIDS patients and patients with malignancies. In all these cases HCMV-Ab negative, patients receiving blood may benefit from HCMV-Ab negative blood although this not standard practice at present.
HCMV infection in transplant patients can be reduced by serological screening of donors, patients and transfused blood and products. However, this approach will not afford any protection against congenital infection or infection due to reactivation in a previously infected individual. In these situations, more formal approaches of antiviral therapy and vaccination are necessary.

1.7.1 Antiviral Therapy

Antiviral therapy is of two types; high titre immunoglobulin (HT-Ig), and specific antiviral drugs.

1.7.1.1 Antiviral Drugs

A number of antiviral agents have been used for prophylaxis and for the treatment of established HCMV infections. Currently, ganciclovir appears to be the most promising antiviral agent (Collaborative DHPG Study Group, 1986), and recently, the combined use of ganciclovir and HT-Ig has been found to be even more effective in the treatment of established infections (Bratanow et al, 1987; Schmidt et al, 1988). Other agents that have been used include acyclovir (Meyers et al, 1988), foscartern (Farthing et al, 1987) and α-interferon (α-IFN) (Hirsch et al, 1983).

1.7.1.2 HCMV Prophylaxis

Ganciclovir, acyclovir and α-IFN have all been used prophylactically with varying results. α-IFN has been used inconclusively in a number of studies. Hirsch et al (1983) reported its effectiveness in preventing HCMV disease in renal allograft recipients, whilst the results of a
number of other studies (Weiner et al, 1978; Cheeseman et al, 1979; Kramer et al, 1984; Kovarik et al, 1988) led to the conclusion that α-IFN had no prophylactic action. Acyclovir, at high dose levels, has been shown to provide some degree of protection against HCMV infection in bone marrow transplant recipients (Gluckman et al, 1983; Meyers et al, 1988), though Pettersson et al (1985) found it ineffective for renal transplant recipients. The value of ganciclovir prophylaxis is as yet unclear, although studies are currently being undertaken to assess its effectiveness.

1.7.1.3 Treatment of Established HCMV Infection

Ganciclovir, acyclovir, α-IFN and foscarnet have all been used in the treatment of established HCMV infection. α-IFN therapy has proven ineffective in the treatment of established HCMV pneumonitis, both on its own (Meyers et al, 1980) or in combination with vidarabine (Meyers et al, 1982), or acyclovir (Wade et al, 1982).

Foscarnet has been successfully used in the treatment of HCMV disease in AIDS patients (Farthing et al, 1987; Weber et al, 1986) and renal allograft recipients (Ringden et al, 1986), and of pneumonitis in bone marrow allograft patients (Apperley et al, 1987). However, high levels are required and there has been concern over possible irreversible nephrotoxicity and deposition of the drug in bone and cartilage.

Acyclovir has been found to have variable efficacy for the treatment of HCMV disease. Balfour et al (1982) concluded that high dose therapy was effective in certain patient groups, especially renal allograft recipients.
Ganciclovir has been found to provide the most consistent antiviral activity against HCMV infection, proving effective in the treatment of HCMV retinitis in AIDS patients (Jennens et al, 1990; Laskin et al, 1987), and HCMV disease in renal allograft recipients (Hiesse et al, 1989) and in heart and heart/lung transplants (Keay et al, 1987). The levels of drug required for the inhibition of HCMV growth are much lower than those for acyclovir and foscarin, but renal excretion is rapid, approximately 99% of the ganciclovir administered is excreted unmetabolised by the kidneys. Ganciclovir has significant toxicity for all groups of patients, neutropoenia being the most common finding (Laskin et al, 1987). The neutropoenia is completely reversible, normal levels returning within a few days of ceasing therapy (Buhles et al, 1988). Recent studies have suggested that the use of ganciclovir together with HT-Ig is very effective for controlling HCMV infection (Reed et al, 1987a; Bratanow et al, 1987).

1.7.2 Use of High Titre Immunoglobulin (HT-Ig)

Hyperimmune globulin (HT-Ig) therapy has been shown to reduce the severity of HCMV disease in allograft recipients, but its overall value in antiviral therapy remains unproven. Winston et al (1982) and Blacklock et al (1985) both reported a decrease in the incidence of HCMV pneumonitis in marrow allograft recipients following HT-Ig treatment, whilst Reed et al (1987b), Bowden et al (1986) and Kasiske et al (1989) found that the use of HT-Ig had no effect on the incidence of HCMV infection after marrow and renal allografts respectively. However, comparisons of studies reporting the use of HT-Ig have to be made with caution due to inherent differences in the products, as well as in the treatment regimens used. Currently, it
would appear that the value of HT-Ig in the treatment of HCMV infections lies in its use in combination with ganciclovir.

1.7.3 Vaccination

The production of a vaccine against HCMV has been an area of interest for a number of years. Two approaches have been used; live (attenuated) vaccines and peptide sub-unit vaccines.

1.7.3.1 Live Vaccine

The first use of a prototype live vaccine was reported by Elek & Stern (1974) who immunised 26 adult volunteers with a preparation of the laboratory strain AD169. Within eight weeks, 25 of these individuals had developed antibodies reactive against the virus. None of the recipients were found to be excreting virus. Initially the production of antibodies recognising other strains of HCMV was limited, but one year after vaccination successfully vaccinated individuals were found to have neutralising antibodies to all strains of HCMV tested. Plotkin et al (1976) obtained similar results using a vaccine prepared from the Towne strain of HCMV. Plotkin et al (1984) vaccinated a group of 91 renal allograft recipients together with a control group of healthy individuals. No excretion of virus was detected, and cellular and humoral immune responses developed in most vaccinees although Ab titres were lower in transplant recipients compared to the control group. They concluded that without vaccination, half of the recipients at risk of acquiring HCMV disease would have developed severe illness.

The effectiveness of vaccination against HCMV was further studied by Plotkin et al (1985) who challenged normal and vaccinated individuals
with low passage Toledo strain of HCMV at doses of 10-100pfu. The vaccinees were found to be protected whilst all of the unvaccinated HCMV-Ab negative individuals developed infection. None of the HCMV-Ab positive unvaccinated individuals developed infection at those doses of virus, although challenge with 1000pfu did induce infection. Further challenge studies (Plotkin et al, 1989) confirmed these findings and showed that at one year post vaccination, the vaccinees possessed immunity to HCMV as complete as natural immunity.

The production of neutralising Ab after vaccination with the Towne vaccine was reported by Gonczol et al (1986, 1989). This group further showed that the differences in neutralising titre were not related to differential detection of reactivity against any HCMV-specific proteins demonstrated by immunoblotting.

1.7.3.2 Peptide Subunit Vaccines

Studies undertaken to identify viral proteins of importance in protective immunity indicated that the gA/gB glycoprotein complex (Gonczol & Plotkin, 1990) was recognised by all sera with neutralising activity. This gA/gB envelope complex consists of a number of glycoproteins previously described independently by other workers: gA (Pereira et al, 1982a), gp55-116 (Britt et al, 1988), and gB (Cranage et al, 1986). The potential of the gA/gB complex for the induction of immunity has been demonstrated by Gonczol et al (1990). Purified gA/gB complex was given to two HCMV-Ab positive and three HCMV-Ab negative individuals, every two weeks. The HCMV-Ab positive recipients responded after the first dose of complex by developing higher titres of neutralising Ab and a transient increase in LPR. The HCMV-Ab negative recipients did not produce Ab until after the third
dose, and the response was only transient. After the fourth dose there was a rapid reappearance and rise in titre of Ab which then persisted. The Ab level one year after the primary dose in previously HCMV-Ab negative individuals was comparable to that seen in naturally infected individuals. Gonczol & Plotkin (1990) concluded that the purified gA/gB complex induces both cellular and humoral immune responses and may serve as the basis of a subunit vaccine.

1.8 STUDY OBJECTIVES

The objective of this investigation was to study natural HCMV infection in blood donors. The approaches taken have been; to study the excretion of virus by healthy individuals, to attempt to demonstrate the presence of virus or viral nucleic acid in donated blood, and to validate the use of serology to identify those donations at risk of transmitting HCMV.

The practical aspects of the study fall into two main parts. First, the investigation of the current prevalence of infection in the donor population, as measured by the presence of circulating HCMV-Ab, the relationship between serological status, age and sex, the expected and actual serocoverson rates, and the incidence of post-transfusion HCMV infection in the UK. Qualitative and quantitative aspects of the immune response to HCMV were further investigated by immunoblotting.

Second, molecular techniques have been used, in conjunction with conventional methods, to identify virus or viral nucleic acid in both the plasma and cellular fractions of donated blood and secretions of normal individuals, and to determine the significance of any such
findings. Furthermore, in the light of new technology, to assess the continuing validity of HCMV-Ab testing to identify those donations at risk of transmitting HCMV.

The immune response to HCMV has been studied by the use of immuno-blotting techniques. Qualitative and quantitative aspects of the immune response have been studied to investigate any correlation between Ab titre, specific protein profile, and long term stability of the immune response. The use of HCMV-Ab status in the provision of blood selected for 'at risk patients' has led to a reliance on the HCMV-Ab response as a marker of previous infection although there is only limited data on the stability of the immune response to HCMV.

Although the presence of HCMV-Ab is generally accepted as an indication of potential infectivity, the direct evidence for this is limited. Other markers such as viruria (Kane et al, 1975) and the presence of HCMV-IgM (Tegtmeier, 1986) have been proposed as potential indicators of infectivity; the assumption being that the donor had been recently infected with HCMV, perhaps with continued virus replication, and thus may present a significant risk of infection.

The increasing availability and use of more sensitive techniques and new technologies, notably molecular techniques, has facilitated the acquisition of more detailed data, especially concerning the sites of virus latency (Schrier et al, 1985; Stanier et al, 1989).

This investigation has been designed to provide a comprehensive study of HCMV infection in healthy individuals; including the incidence, significance, outcome, and implications of infection in blood transfusion practice. The provision of this information was primarily through the collection of accurate and current data on the prevalence
of HCMV in the donor population served by the N.E. Thames Regional Transfusion Centre, and importantly, the prevalence of HCMV infection in the general population, as measured by the observed seroconversion rate in the donor population. To develop this information, DNA hybridisation has been used to identify donors shedding virus into the blood, urine or saliva, and also to detect HCMV NA sequences that may be present in PBMC. This last aspect has been complemented by the use of PCR with its ability to detect specific DNA or RNA sequences present in tissue at a very low copy number (Saiki et al, 1988).
CHAPTER 2  MATERIALS AND METHODS

2.1  STUDY POPULATION

The study population comprised blood donors attending routine donor sessions to donate a normal unit of whole blood, donors attending the apheresis unit at the Brentwood Regional Transfusion Centre either for platelet or plasmapheresis, and members of staff who, although formal donation of blood was not undertaken, at the time of sampling fulfilled the criteria necessary to donate blood. All the individuals in these groups were considered as healthy donors.

Venous blood samples were obtained from normal donors as part of the routine donation procedure, and from staff members by special collection.

2.1.1  Serum Samples

Blood samples were stored at 4°C overnight prior to separation of the serum. Separated serum was either used immediately or stored at -40°C prior to use.

2.1.2  Leucocyte Samples

Peripheral blood mononuclear cells (PBMC) were separated from EDTA anticoagulated whole blood samples by density gradient centrifugation on a ficoll/triosil gradient (Appendix). The cells were either used immediately or stored at -40°C prior to use.

2.1.3  Urine Samples

Urine samples were obtained only from the groups of apheresis donors and staff members. Donors were asked to collect their complete, early
morning urine specimen, as aseptically as possible; a minimum volume of 250ml being required. Samples for culture were separated immediately and stored in liquid nitrogen. The remainder was either processed within twelve hours of collection or stored at -40°C for up to one month prior to processing.

2.1.4 Saliva Samples

Saliva samples were obtained from those individuals who provided urine samples and at the time of urine collection. Donors were asked to provide a small volume, minimum 5ml, of normal saliva, collected over a period not exceeding 24 hours. Samples were either processed immediately or stored at -40°C for up to one month prior to processing.

2.1.5 Donor Details

Information was obtained regarding the donor’s age (at the time of donation), sex, HCMV-IgG status, and where applicable, previous HCMV-IgG status. To facilitate statistical analysis, donors were grouped according to age in intervals of five years, except for the youngest age group, in which the age group was three years: 18-20, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, 61-65 years.

The data were then used to determine: the overall prevalence of HCMV, as measured by the presence of HCMV-IgG, in a healthy blood donor population; the relationships between age, sex and HCMV-IgG status; the relationships between age, sex and HCMV-IgG titre; the relationships between age and the observed seroconversion rates; and the correlation between the observed and expected seroconversion rates.
2.1.6 Seroconversion of HCMV-Ab Negative Donors

The seroconversion rate of HCMV-Ab negative donors was determined by monitoring the subsequent donations of those HCMV-Ab negative donors initially bled during the period from 01/01/87 to 30/6/88, until 30/6/90. Donors were only included in the study if subsequent donations were made over a period of at least one year. The complexity of the donor call-up system, the reluctance to make too many extra demands on donors, the great variability in the demand for HCMV screened blood and blood products, and the subsequent rather erratic screening regime all resulted in difficulties in gathering and maintaining a panel of donors for a full prospective study. Seroconverted donors were identified by conversion from HCMV-Ab negative to HCMV-Ab positive during the study period. The serostatus of any seroconverted donors, both pre- and post-seroconversion, was confirmed, where possible, by repeat testing of any archived serum samples available.

2.1.7 Statistical Methods

Data were analysed using the following statistical formulae:

Chi-squared analysis (2x2 contingency table) with Yates' correction

\[ \chi^2 = \frac{n(\frac{1}{4}ad-bc-\frac{1}{2}n)^2}{(a+b)(c+d)(a+c)(b+d)} \]

Used to analyse the prevalence of HCMV-IgG in male and female donors.
Chi-squared analysis (general rxc contingency table)

\[ \chi^2 = \sum \frac{(O-E)^2}{E} \]

Used to analyse the range of HCMV-IgG titres in male and female donors.

Correlation coefficient

\[ r = \frac{\sum (x-x)(y-y)}{\sqrt{\sum(x-x)^2 \sum(y-y)^2}} \]

Student's' t-distribution (n-2 df)

\[ t = \frac{r(n-2)}{\sqrt{1-r^2}} \]

Used to determine the correlation between the HCMV-IgG titre and donor age, the null hypothesis being that there is no significant association between HCMV-IgG titre and the age of the donor.

2.2 DETECTION OF HCMV-SPECIFIC ANTIBODY

2.2.1 Routine Screening of Donors

Serum samples from normal healthy blood donors were screened for the presence of HCMV-IgG using standard commercial assays, CMV Total Antibody (Abbott Diagnostics), Comp Enz C (Northumbria Biologicals), Captia G (Mercia Diagnostics), and Bioelisa CMV-G (Launch Diagnostics). Samples were selected either at random for investigative
purposes, or on the basis of ABO and Rhesus(D) blood groups when HCMV screened blood and blood products were required for clinical use. All assays were performed in accordance with the manufacturers' instructions, validated according to their validation criteria and the results calculated accordingly. A number of donors screened as HCMV-IgG positive were deemed to be high titre by reference to a standard antibody preparation (ACMV01) produced by the Central Blood Laboratories Authority (CBLA) Elstree, Herts, and tested with each batch of routine HCMV-IgG screening. This preparation is produced specifically as a reference against which antibody levels in normal donors can be compared and represents the minimum antibody level acceptable for use in the production of HCMV immunoglobulin (HCMV-HT Ig). Plasma packs from donors with high titre HCMV-IgG were consequently used specifically for HCMV-HT Ig production.

2.2.2 HCMV-IgM Antibody Screening

HCMV-IgM was detected using the Captia M HCMV EIA (Mercia Diagnostics), in combination with an 'in house' HCMV-IgM EIA. Donor serum samples were initially screened for the presence of HCMV-IgM using the 'in house' IgM assay; positive samples were then retested using a commercial HCMV-IgM assay (Captia M, Mercia Diagnostics). The assay was performed in accordance with the manufacturers' instructions and samples initially reactive by the assay were repeated. If these were repeatedly reactive, they were considered to be HCMV-IgM positive. Serum and PBMC samples were saved from all donors reactive by the 'in house' IgM assay and stored at -40°C until the IgM status was confirmed.
2.2.3 HCMV-IgA Antibody Screening

Donor serum samples were screened for the presence of HCMV-IgA using an 'in house' EIA. Samples were selected on the basis of their HCMV-IgG status. Serum and PBMC samples were saved from HCMV-IgA positive donors and were stored at -40°C.

2.2.4 HCMV-Specific 'In House' EIAs

'In house' HCMV enzyme immunoassays (EIAs) were developed to detect HCMV-IgG, -IgM and -IgA, the assay preparation and format being identical for all three isotypes. The substrate/detection system was subsequently modified in the IgA assay to increase sensitivity and specificity.

The IgG assay was developed to facilitate cheap, mass screening of donors; the IgM and IgA assays were developed to investigate the prevalence of HCMV-IgM and -IgA in blood donors.

2.2.4.1 Preparation of Antigen Coated Wells

Plastic micro-wells, Nunc U-16 Maxisorb 'Remova wells' (Gibco BRL) in the standard 96 well microplate format, were coated with HCMV antigen, EIA grade (Inst. Virion, Flow Labs), diluted 1/175-1/200 in bicarbonate coating buffer pH 9.6 (Appendix). Coating was performed at 4°C for 16 hours in humidified containers, after which the plates were washed with distilled water, thoroughly dried, individually sealed in plastic bags with a desiccant sachet and stored at 4°C for up to six months.
2.2.4.2 Assay Methodology

Test and control sera were prediluted 1/40 in sample/conjugate diluent (Appendix). Diluted test and control sera were added to the appropriate wells and the plate incubated for 30 minutes at 45°C. The plates were then washed three times in wash buffer (Appendix), and appropriately diluted conjugate (peroxidase labelled anti-human IgG, IgM or IgA - Jackson Immunoresearch, USA) added to each well. The plates were incubated at 45°C for a further 20 minutes and then rewashed as above. Substrate (3,3',5,5'-tetramethylbenzidine [TMB], Appendix) was added to each well and the plates incubated for 10 minutes at RT. The reaction was stopped by the addition of 2M sulphuric acid and the absorbance read at 450nm.

The cut-off value for the IgG assay was calculated from the mean of the negative controls, c/o = Ncm x 1.5; the cut-off value for both the IgM and IgA assays was calculated in the same way, from the mean of the positive controls, c/o = Pcm x 0.75.

The HCMV-IgA assay was subsequently modified, by the use of the IQ Bio substrate/amplifier detection system as used by Wellcome Diagnostics in a number of their diagnostic assays. Excess reagents from the Wellcome assays provided this system. The 'in house' assay was used according to the methodology described in section 2.2.3.2 except that an alkaline phosphatase labelled anti-human IgA (Jackson Immunoresearch, USA) was used, and the substrate replaced by the IQ Bio system. After incubation with conjugate, the wells were washed in piperazine buffer (Appendix) and substrate solution (Appendix) was added. The wells were incubated at 45°C for 20 minutes and amplifier solution (Appendix) was added. The wells were incubated in the dark.
at RT for a further 10 minutes and the reaction stopped by the addition of 2M sulphuric acid. The absorbance was read at 492nm. The cut-off value was calculated from the mean of the negative controls, c/o = Ncm x 2.

2.2.4.3 Titration of HCMV-Antibody

HCMV-IgG and -IgM were titrated using the 'in house' assay. Doubling dilutions of test sera were prepared in sample/conjugate diluent and tested using the standard assay methodology. The end-point was determined as the highest dilution giving a positive result.

2.2.4.4 Assessment and Validation of 'In House' EIAs

The IgG assay was validated against a panel of 90 sera also used to assess commercial assays. The IgM assay was validated against a panel of 10 known HCMV-IgM sera, supplied by AE Hardiman, Virology Department, St Bartholemews Hospital, London.

Validation of the IgA assay was based on the IgA IB results. Further validation was not possible due to the absence of either a commercial HCMV-IgA assay or standardised HCMV-IgA sera.

The negative control serum used in all assays was obtained from a single seronegative individual. The IgG assay positive control serum was the reference preparation ACMV01 (CBLA), used to identify high titre donors. This serum was consequently used as both a positive control and a high titre reference sample. The HCMV-IgM assay positive control serum was a selected weak HCMV-IgM serum obtained from the PHLS, Chelmsford, Essex. The HCMV-IgA assay positive
control was a clinical specimen originally obtained on the basis of HCMV-IgM status from St Bartholemews Hospital, London.

2.3 CELL CULTURE

2.3.1 Cell Lines

Cell culture performed using human embryonic lung fibroblasts (HEL cells) produced as an 'in-house' cell line, and supplied on a fortnightly basis by the Virology Department, St Bartholemews Hospital London. Cells were generally grown in 25 or 75cm² plastic flasks, although 120cm² and 200cm² glass flasks were also used.

Cell lines were propagated in Eagle's Minimum Essential Medium (Modified) with Hank's Salts (Flow Labs), containing 1.85gm/L sodium bicarbonate, 2mM glutamine, 100u/ml penicillin (Gibco BRL), 100µg/ml streptomycin (Gibco BRL), 100µg/ml kanamycin (Gibco BRL) and supplemented with either 10% (growth medium) or 2% (maintenance medium) heat inactivated foetal calf serum. The antibiotics penicillin and streptomycin, and the antimycotic kanamycin were subsequently replaced by 100µg/ml Gentamicin (Flow Labs).

Cells were propagated by growing to confluence and splitting 1:2. Confluent cells were washed with phosphate buffered saline (PBS) and treated briefly with 0.25% trypsin/EDTA solution prewarmed to 37°C. The enzyme solution was removed and the cells incubated at 37°C for 2-3 minutes and then gently resuspended in an appropriate volume of growth medium.
2.3.2 Cell Stocks

Whenever possible, stocks of HEL cells were prepared and stored frozen in the vapour phase of liquid nitrogen for up to 12 months.

Confluent cells were trypsinised as described in section 2.3.1, and resuspended in freezing medium, EMEM prepared as described in section 2.3.1 but containing 20% foetal calf serum and 15% dimethyl sulphoxide (DMSO), at a concentration of approximately $10^6$ cells/ml. The cells were aliquoted and frozen slowly in liquid nitrogen vapour.

Cells were recovered by thawing rapidly at 25°C and transferring the cell suspension into a 25cm² plastic flask. 10ml of growth medium was added and the cells incubated for 4–6 hours. The medium was aspirated and replaced with 10ml of growth medium, pre-warmed to 37°C. The cells were examined after 24 hours and the medium changed if necessary. Cells were normally passaged at 72–96 hours.

2.3.3 Virus Stocks

The major part of this study used the prototype laboratory strain of human cytomegalovirus, AD169, as the infectious agent, although later work included two other laboratory strains, Davis and Towne, as well as a small number of clinical isolates.

Virus stocks were prepared, as both culture supernatant and infected cells, by infecting cell monolayers at 80–100% confluence. Cells were infected at a multiplicity of infection (moi) of 0·1–1. The medium was removed and replaced by a minimal volume of maintenance medium containing virus, and incubated at 37°C for 1–2 hours to allow virus adsorption. After incubation, the inoculum was removed and replaced by growth medium. After 24 hours this was replaced by maintenance
medium and the cells were then checked daily for cytopathic effect (cpe). Three to four days after 100% cpe the cells and medium were harvested. All stocks of virus were frozen initially at -40°C and then stored in the vapour phase of liquid nitrogen.

2.3.4 Virus Titrations

Virus stocks were titrated and aliquoted to facilitate optimal infection of the cell lines at a known m.o.i. Cells were grown to confluence in 24 well plates under standard conditions. Virus stocks were diluted tenfold from 10^0 to 10^-6 in maintenance medium. 400μl of each dilution was added to the appropriate wells and the plate was incubated for 1-2 hours to allow adsorption. The inoculum was removed and the cells overlaid with 1·5 ml of maintenance medium/agarose. The medium/agarose solution was prepared by mixing equal volumes of 2x maintenance medium and 2% low melting temperature agarose prepared in culture grade water and cooled to 45°C. The overlay was allowed to set at room temperature and the plate then incubated at 37°C for 1-2 weeks with daily examination after one week. Once a cpe had appeared, up to three weeks maximum, the cells were fixed by flooding the plate with 5% v/v formaldehyde in PBS and incubating overnight at room temperature. The agarose was removed by washing the wells carefully in warm tap water and the cells were stained with 0·5% methylene blue in PBS. The stained, dried plate was examined and the foci counted. Virus titre was then expressed as the number of detectable foci/ml calculated to be present in the undiluted virus stock.
2.3.5 Synthesis of HCMV IE RNA

HCMV IE RNA was prepared either by harvesting HCMV infected cells approximately 12-18 hours after infection, or by specifically increasing the level of IE RNA by blocking α-protein synthesis using cycloheximide.

2.3.5.1 Standard Culture Method

Confluent HEL cells were infected with HCMV at a moi of 1. 12-18 hours after infection the cells were harvested and the RNA extracted using the method of Chomczynski & Sacchi (1987), (see section 2.6.2.5).

2.3.5.2 Cycloheximide Induction

Confluent HEL cells were treated with cycloheximide at 200µg/ml of medium for one hour prior to infection. The cells were infected with HCMV at a moi of 0.1-1 in medium containing 200µg/ml cycloheximide. 24-48 hours after infection the cells were harvested and the RNA extracted using the method of Chomczynski & Sacchi (1987), (see section 2.6.2.5).

2.3.6 Preparation of HCMV Blotting Antigen

Antigen for Western Blotting was prepared as infected or uninfected (control) HEL cell lysate. To standardise the integrity of the cell lysate used as the antigen source, the same cell line at the same passage number was used for each batch of antigen prepared. HEL cell line 42, obtained at passage 12, was grown to passage 15, aliquoted and frozen in liquid nitrogen as previously described. When required for antigen preparation, the cells were recovered and grown to passage 17-18 for use.
HEL cells were grown to 80-100% confluence and infected with the AD169 strain of HCMV at 0·1 moi as described above. Three to four days after 100% cpe, just as cells were starting to detach from the flask surface, the medium was removed and the cells harvested as blotting antigen. The cells were rinsed carefully with PBS and then drained to ensure that no rinse solution remained. Cell lysis buffer (Appendix) was heated in a boiling water bath for 3-4 minutes and then immediately pipetted over the monolayer, 1ml/25cm² of monolayer, with care being taken to ensure that all the cells were harvested. The lysis solution containing the harvested antigen was recovered and transferred to a glass universal and boiled for two to three minutes to ensure complete denaturation of the proteins. The antigen was cooled and stored at -40°C. Control antigen was prepared in the same way from uninfected HEL 42 cells at the same passage.

2.3.7 Propogation of Other Herpesviruses

Isolates of the other members of the human herpesvirus family were propagated, primarily to provide DNA and RNA for use as specificity controls for dot-blot and PCR analysis.

Herpes simplex I (HSV I), Herpes simplex II (HSV II) and Varicella Zoster (VZV) were obtained from T. Lewis, Public Health Laboratory Service, Chelmsford, Essex and propagated in HEL cells. Epstein Barr virus (EBV) was obtained from P. Farrell, The Ludwig Institute of Cancer Research, St Mary's Hospital Paddington, London and propagated in the Raji B lymphocyte cell line. Human Herpesvirus 6 (HHV-6) was obtained from R. Honess, The National Institute for Medical Research, Mill Hill, London and propagated in the J.Jahn B lymphocyte cell line.
2.4 IMMUNOBLOTTING

2.4.1 Preparation of HCMV Antigen

Antigen for Western Blotting was prepared as described in section 2.3.6. Antigen was also prepared in the same manner from the laboratory strains Towne and Davis, as well as from clinical isolates.

2.4.2 Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were prepared according to standard protocols (Appendix). Gels were poured externally to the electrophoresis apparatus (Gradiphore) and slotted into position in the tank for use. Gels were stored at 4°C for at least 24 hours prior to use, but not for longer than five days after preparation. Initially, gels were cast as 3mm thick gels, 75mm wide and 120mm long, consisting of 9% resolving gel and 4% stacking gel. Subsequently, 1mm thick gels were prepared and used, although problems were encountered in stabilising the current flow in the tank, occasionally resulting in variable migration patterns.

The prepared gels were loaded into the electrophoresis tank (maximum 2 gels) and allowed to equilibrate with running buffer (Appendix) for 30 minutes. Approximately 350μg of antigen was loaded onto the gel (50μg/cm) and approximately 30μg of molecular weight marker solution (SDS-6H, Sigma) was loaded into the marker lane. 3mm gels were run at a constant current of 80mA for 5-6 hours, and 1mm gels were run at a constant voltage of 120 volts with a current limit of 20mA for 4-5 hours. Both types of gel were run until the dye front was approximately 5mm from the bottom of the gel.
The exact distance of the dye front from the origin was measured and recorded and then the lane containing the protein markers was cut from the gel prior to transfer. The strip was stained with Coomassie Blue for 2-3 days and then destained for 1-2 days (Appendix). The distances migrated by the individual protein markers were measured and the relative mobilities calculated. A reference curve of molecular weight v. relative mobility was then plotted.

2.4.3 Transfer

Protein transfer was performed using a Trans Blot cell (Bio Rad) to electrophoretically transfer the resolved proteins onto nitrocellulose membrane (BA 85, Schleicher & Schuell). Transfer was performed at 210mA for 3-4 hours at 20°C in standard transfer buffer (Appendix), after which the membranes were rinsed briefly in distilled water and air dried for 30 minutes at room temperature. The membranes were then blocked with 1% dried skimmed milk (Marvel) in PBS for two hours at room temperature, rinsed again in distilled water, dried thoroughly and finally cut into 2-3mm wide strips with the orientation, origin and dye front marked, wrapped in foil and stored at -40°C.

2.4.4 Immunoblotting Procedures

2.4.4.1 Primary Incubation

Reactions were performed in dedicated reaction trays in a reaction volume of 1ml for each stage of the assay and with constant agitation. Strips were pre-incubated in blocking solution (Appendix) for 1-2 hours. This was replaced with 1ml of reaction buffer (Appendix), and the strips incubated for a further 15-30 minutes. 20μl of test serum was added and the strips incubated overnight at room temperature.
2.4.4.2 Detection and Visualisation

The strips were washed three times in TBTS (Appendix), 20 minutes per wash, and then incubated for two hours at room temperature in 1ml of an appropriate dilution of biotin labelled, anti-human IgG, IgM or IgA (Vector Labs.) in antibody buffer (Appendix). The strips were again washed three times in TBTS, 20 minutes per wash, and then incubated for a further two hours in 1ml of an appropriate dilution of extravidin peroxidase (Sigma) or streptavidin peroxidase (Jackson Immunoresearch) in antibody buffer. The strips were finally washed once for 20 minutes in TBTS and then twice in TBS (Appendix), 20 minutes per wash. The reaction products were visualised by the addition of 0.5mg/ml 4-chloro-1-napthol (Bio Rad) in TBS/Methanol (Appendix). After colour development, the strips were rinsed in distilled water, fixed in 25mM sulphuric acid and then rinsed again in distilled water. The strips were dried and mounted on 3MM paper.

2.4.4.3 Donor Samples

Immunoblotting was performed on serum samples from HCMV-IgG positive and negative donors. The specificity of the assay was demonstrated using a panel of sera containing high titre antibodies to the other human herpesviruses, but negative for antibodies to HCMV. Serum samples giving repeatedly equivocal results with commercial assays, or discrepant results between assays, were also investigated in an attempt to determine the true HCMV-specific antibody status of these donors.
2.5 NUCLEIC ACID BIOCHEMISTRY

2.5.1 Preparation of Probes

An HCMV-specific DNA probe was prepared from a cloned HCMV DNA fragment excised from pCM5018 which contains the 10.6kb HCMV AD169 Eco RI J fragment (obtained from B Fleckenstein, Universität Erlangen, Erlangen FRG).

Clone pBP01 was used to generate RNA transcripts from the immediate early region of HCMV AD169 DNA and was produced by cloning the Pst I w fragment of HCMV AD169 (Greenaway et al, 1982) into the Bluescript+ transcription vector (Stratagene, Northumbria Biologicals).

Clone pCM01 was used to produce DNA fragments with the suitable restriction sites for cloning into the bacteriophage vectors M13mp18/mp19 prior to DNA sequencing. The clone was produced by cloning the specific reaction product from HCMV RNA PCR into pUC19.

2.5.1.1 Preparation and Purification of Plasmid pCM5018

The plasmid was propagated and purified using standard procedures. Cells were grown overnight in one litre cultures of LB medium (Appendix) containing the appropriate antibiotic. The cells were lysed using the 'alkaline lysis' method (Birnboim & Doly, 1979; Ish-Horowicz & Burke, 1981), and the plasmid purified by caesium chloride-ethidium bromide density gradient centrifugation (in a Beckman VTi80 rotor run at 50,000 rpm for 16 hours at 20°C). Following removal of the ethidium bromide and caesium chloride from the DNA solution, the DNA was recovered by ethanol precipitation and the purified plasmid was resuspended in TE pH 8.0 (Appendix) and stored at 4°C. The concentration of the plasmid was calculated by measuring the
absorbance at 260nm ($A_{260}$), and the purity estimated by calculating the $A_{260}/A_{280}$ ratio.

The HCMV specific fragment was prepared for use as a hybridisation probe by digesting the plasmid Eco RI and separating the insert and vector by agarose gel electrophoresis. The agarose containing the insert was cut from the gel, the DNA electroeluted from the gel, purified on DEAE-Sephacel (Pharmacia - Appendix), and resuspended in TE pH 8.0. The concentration of the purified fragment was estimated by running aliquots on a 1% agarose/TBE gel (Appendix) together with 1µg of Hind III digested bacteriophage λ DNA, and comparing the intensity of the fluorescence of the specific fragment with the intensity of the known mass of control DNA fragments. The purified fragment was stored frozen at -40°C.

2.5.1.2 Preparation and Purification of Plasmid pBP01

pBP01 was produced by cloning the Pst I w fragment of HCMV AD169 (Greenaway et al, 1982) into the RNA transcription vector Bluescript+ (Stratagene, Northumbria Biologicals).

Plasmid pCM5018, containing the Eco RI J fragment of HCMV AD169, was digested with Pst I and the resulting fragments separated on a 1% agarose/TAE gel (Appendix). The 1487bp, Pst I w fragment was excised from the gel and purified using the 'Gene Clean' glass milk procedure (Bio 101, Stratech Scientific). The fragment was ligated into Pst I linearised Bluescript+.

TG-2 cells (Appendix) were made competent using the CaCl₂ method of Mandel et al (1970) and then transformed by incubation on ice for 30 minutes with the ligated DNA. Transformants were selected on the
basis of formation of Amp\textsuperscript{*} white colonies when grown in the presence of IPTG and X-gal (Appendix). The colonies were picked out onto fresh IPTG/X-gal plates and confirmed transformants plated out.

Overnight cultures, in 25ml of LB medium, were grown from the selected transformants and the plasmids harvested using a rapid lysis technique and digested with Pst I to check for the presence of the cloned insert. Clones containing the desired insert were then grown in LB medium and bacterial stocks prepared in 15% glycerol and stored frozen at -40°C.

One such plasmid, pBP01, was propagated in TG-2 cells. Cells were grown overnight in 500ml cultures of Circlegrow medium (Bio 101, Stratech Scientific) containing ampicillin. After harvesting, the cells were lysed by a modification of the alkaline lysis method and the plasmid purified by spun column chromatography using the pZ523 column (5 Prime\textsuperscript{*}3 Prime Inc.). The purified plasmid was resuspended in TE pH 8.0 and stored at 4°C. The concentration of the DNA was calculated as described above by measuring the A\textsubscript{260} and the A\textsubscript{260}/A\textsubscript{280} ratio.

2.5.1.3 Preparation and Purification of Plasmid pCM01

pCM01 was produced by cloning the HCMV-specific product from RNA PCR performed on RNA prepared from PBMC (section 2.5.6.3), into pUC19.

The 227bp product from RNA PCR was purified from the remaining stored PCR reaction mixture by extracting the oil overlay with 200\textmu l of chloroform and then extracting the aqueous layer twice with an equal volume of phenol/chloroform. The DNA was then precipitated by the
addition of 0.1 volume of 3M sodium acetate and 2.5 volumes of absolute ethanol.

The purified fragment was blunt-end ligated into Sma I digested pUC19 using T4 DNA and RNA ligases for 16 hours at 12°C. TG-2 cells were made competent, transformed and recombinants identified as described in section 2.5.1.2.

The resultant plasmid, pCM01, was propagated in TG-2 cells. Cells were grown overnight in 150ml cultures of Circlegrow medium (Bio 101, Stratech Scientific) containing ampicillin. After harvesting, the cells were lysed by a modification of the alkaline lysis method, the plasmid purified by column chromatography using Qiagen pack 100 columns (Hybaid), resuspended in TE pH 8.0 and stored at -40°C.

2.5.1.4 Preparation of Sequencing Templates

Purified plasmid pCM01 was 'double-digested' with restriction enzymes Eco RI and Hind III, the fragments separated on a 1.5% agarose gel and the insert excised and purified using DEAE-Sephacel, as described in section 2.5.1.1. The purified fragment was ligated into both M13mp18 and M13mp19, Hind III/Eco RI linearised RF DNA. TG-2 cells were made competent and transformed as described in section 2.5.1.2, except that YT medium (Appendix) was used instead of LB medium. Recombinants were identified as a colourless plaques of slow growing TG-2 cells on a lawn of normally growing cells. Plaques were picked into YT medium containing fresh TG-2 cells, and grown for five hours. The cells were pelleted and the bacteriophage in the supernatant precipitated using PEG (Appendix). The DNA templates were prepared from the PEG pellet
by phenol extraction and EtoH precipitation and then resuspended in TE pH 8.0 and stored at -40°C.

2.5.1.5 Sequencing Reactions

DNA sequencing was performed using the Sanger 'dideoxy method' (Sanger et al, 1977), as modified in the Sequenase (Ver 2.0) (USB Corp) sequencing system, according to the manufacturer’s protocol. Single stranded templates prepared in section 2.5.1.4 above were annealed to an oligonucleotide primer, labelled with [α-35S]dATP and terminated with dideoxynucleotides. The reactions were double-loaded onto a 7% 0.2mm urea/acrylamide gel (Appendix), each loading representing 1/5 vol of the sequencing reaction. The first loading was run until the leading dye front reached the bottom of the gel. The second loading was then run until the leading dye front was 3/4 of the way down the gel. The gel was layered onto 3MM paper, vacuum dried at 80°C for an hour and exposed to Kodak XAR-5 X-ray film at room temperature overnight.

2.5.2 Labelling of DNA Probes

2.5.2.1 Labelling of Double Stranded DNA Probes

Double stranded DNA probes were labelled with 32P by the random primer method of Feinberg and Vogelstein (1983), using the large fragment of DNA polymerase I (Klenow fragment) with random oligodeoxyribonucleotides (hexamers) as primers. 50ng of DNA was labelled in reactions containing 50μCi of [α-32P]dCTP at approximately 3000Ci/mmol (Amersham, PB10205). After labelling, the reaction was stopped by the addition of 0.5M EDTA and the DNA was purified by spun column chromatography through Sephadex G50. The specific
activity was estimated by TCA precipitation of an aliquot of the DNA onto glassfibre filters. The labelled DNA was stored at -40°C for up to a maximum of 10 days prior to use.

2.5.2.2 Labelling of Oligonucleotide Probes

Oligonucleotide probes CM.PR1 and CM.PR2 were labelled with $^{32}$P at their 5' ends using T4 polynucleotide kinase. 200ng of oligonucleotide was labelled per reaction using 50μCi of [α-$^{32}$P] ATP at approximately (Amersham, PB10168). After labelling, the reaction was stopped by heating the reaction mixture to 75°C for 15 minutes and the DNA purified by spun column chromatography through sephadex G25 (NAP 25 columns, Pharmacia). The labelled oligonucleotides were stored at -40°C for up to 48 hours prior to use.

2.5.3 Hybridisation

2.5.3.1 DNA/DNA and DNA/RNA Hybridisation

Filters were thoroughly wetted with 6x SSC (Appendix) and then sealed in polythene bags prior to hybridisation. Prehybridisation was performed for 12-30 hours at 37°C in a solution containing 50% formamide (Appendix), 6x SSC, 10mM EDTA, 5x Denhardt's solution (Appendix), 0.5% SDS and 10μg/ml Salmon Testes DNA (Sigma), using a volume of 200μl/cm² of filter. Hybridisation was performed for 18-22 hours at 37°C in prehybridisation solution containing approximately $10^6$ cpm/ml of $^{32}$P labelled DNA probe, using a volume of 50μl/cm² of filter.

Filters were washed once in 2x SSC/0.5% SDS for 5 minutes at room temperature, once in 2x SSC/0.1% SDS for 15 minutes at room
temperature, and then 5-6 times for 60 minutes each at 60°C in 3x
SSC.

2.5.3.2 Oligonucleotide Probe Hybridisation

Filters were sealed in polythene bags prior to hybridisation. Prehybridisation was performed for 4 hours at a temperature 5°C less than the annealing temperature, calculated according to the standard formula of 4°C per C/G base and 2°C per A/T base (Itakura et al, 1984). Prehybridisation was performed at 47°C for oligonucleotide CMP.1 and 53°C for oligonucleotide CMP.2, in a solution containing 5x SSPE (Appendix), 5x Denhardt’s solution, 0.5% SDS and 10μg/ml salmon testes DNA using a volume of 200μl/cm² of filter. Hybridisation was performed for 8-10 hours at the same temperature in prehybridisation solution containing 20ng/ml ³²P labelled oligonucleotide probe, using a volume of 50μl/cm² of filter. The filters were then washed three times in 2x SSPE/0.1% SDS for 15 minutes at room temperature followed by one wash in 2x SSPE/0.1% SDS for 5 minutes at 42°C.

2.5.3.3 Standardisation of Hybridisation Procedures

Hybridisation procedures were standardised by the use of serial dilutions of target DNA, either on filters carrying test sample DNA, or prepared as separate test strips. DNA, in TE pH 8.0 containing 10μg/ml salmon testes DNA (Sigma), was denatured with 0.3M NaOH for 10 minutes at 37°C, cooled on ice and neutralised by the addition of 0.12M HCl (final concentrations). Standard amounts of DNA, 50, 20, 10, 5, 2, 1, 0.5pg, were then applied to nitrocellulose (BA 85, Schleicher &
Schuell) or nylon (Hybond N, Amersham) membranes using a vacuum manifold and hybridised as described above.

2.5.4 Autoradiography

Hybridised filters were air dried, mounted on 3MM paper and wrapped in clingfilm. They were then exposed to X-ray film (Kodak XAR-5) in autoradiography cassettes with intensifying screens (Kodak or Du Pont Cronex) at -40°C for 4-48 hours.

The films were processed manually using 'Suprol' developer and 'Superamfix' fixer (Champion Photochemistry), both at a dilution of 1/4, for 2-3 minutes at room temperature in each solution.

2.5.5 Preparation of RNA Transcripts

2.5.5.1 Template Preparation

The orientation of the cloned insert in pBP01 was determined by restriction digestion of pBP01 by Sma I. The plasmid was linearised downstream of the cloned fragment and the T7 polymerase binding site by digestion with Apa I, or downstream of the cloned fragment and the T3 polymerase binding site by digestion with Xba I. The restriction endonuclease was digested using proteinase K and the linearised template purified by phenol/chloroform extraction and ethanol precipitation and then resuspended at a concentration of approximately 1μg/μl in DEPC treated TE pH 7.5 (Appendix).

2.5.5.2 Transcription Reaction

Sense (T7) and antisense (T3) transcripts were produced from pBP01 for use as PCR substrates and as RNA hybridisation probes.
Unlabelled RNA transcripts were produced from 1µg of template using T3 or T7 polymerases in a reaction mixture containing: 40mM Tris pH 8.0, 8mM MgCl₂, 2mM spermidine, 50mM NaCl, 400µM each of rATP rCTP rGTP rUTP, 30mM DTT, 1u Inhibit-Ace (5 Prime*3 Prime Inc.) and 10u of T3 or T7 polymerase.

Biotin labelled RNA transcripts were produced as described above, except that rUTP was replaced by allylamine-UTP (Enzo Diagnostics). The allylamine-RNA produced was then biotinylated in a separate reaction as described in section 2.5.5.3.

2.5.5.3 Biotinylation of Allylamine-RNA

Allylamine-RNA was biotinylated chemically using ε-caproylamidobiotin-N-hydroxysuccinimide ester (CAB-NHS ester, Gibco-BRL) according to the method of Leuhrs en et al (1987). The allylamine-RNA was resuspended in 0.1M sodium borate pH 8.5 and five volumes were mixed with one volume of 10mg/ml CAB-NHS ester dissolved in N,N-dimethylformamide. The reaction was incubated at room temperature for two hours and the labelled RNA recovered by ethanol precipitation. The RNA was resuspended in DEPC treated TE pH 7.5. Prior to use the probe was further purified by column chromatography using a Select-B(RF) column (5 Prime*3 Prime Inc.) and then stored at -40°C.

2.5.5.4 Hybridisation of RNA Probes

Hybridisation was carried out according to the method supplied with the 'Blu Gene' Nonradioactive Nucleic Acid Detection System (Gibco BRL). Filters were thoroughly wetted in 2x SSC and sealed in polythene bags prior to hybridisation. Prehybridisation was performed for 6-12 hours at 42°C in a solution containing 50% formamide, 5x SSC,
5x Denhardt’s solution, 25mM sodium phosphate (pH 6.5), 0.5mg/ml salmon testes DNA, using a volume of 100µl/cm² of filter. Hybridisation was performed for 18-22 hours at 42°C in a solution containing: 45% formamide, 5x SSC, 1x Denhardt’s solution, 20mM sodium phosphate (pH 6.5), 0.2mg/ml salmon testes DNA, 5% dextran sulphate and approximately 200µg/ml biotin labelled RNA probe.

Filters were washed twice in 2x SSC/0.1% SDS for 3 minutes at room temperature, twice in 0.2x SSC/0.1% SDS for 3 minutes at room temperature, twice in 0.16x SSC/0.1% SDS for 15 minutes at 50°C, and finally rinsed briefly in 2x SSC at room temperature.

2.5.5.5 Visualisation of Hybrids

The washed filter was rinsed in 'Blu Gene' wash buffer (Appendix) for 1 minute at room temperature. The filter was then sealed in a polythene bag and blocked in 'Blu Gene' blocking buffer (Appendix) for 60 minutes at 65°C; incubated in a solution containing streptavidin-alkaline phosphatase (SA-AP) conjugate (1µg/ml) in wash buffer, for 10 minutes at room temperature with gentle agitation; washed twice in a large excess of wash buffer for 15 minutes at room temperature; washed once in Blu Gene substrate buffer (Appendix) for 10 minutes at room temperature; resealed in a polythene bag and incubated for 15-60 minutes in substrate solution (Appendix). The reaction was finally stopped by washing in stopping solution (Appendix) for 2 minutes at room temperature after which the filters were dried and mounted and stored in the dark.
2.5.6 Polymerase Chain Reaction

The polymerase chain reaction was performed on both DNA (DNA-PCR) and RNA (RNA-PCR). DNA-PCR was performed on purified viral DNA, cloned DNA, infected cultured cells, PBMC genomic DNA, and DNA isolated from urine and saliva samples; RNA-PCR was performed on pBP01 RNA transcripts and total cellular RNA from infected cultured cells and PBMC.

2.5.6.1 Oligonucleotide Primers

The oligonucleotide primers used for DNA PCR were 20 mer primers designated CMP1 and CMP2. The sequences (Table 2.1) are located in the immediate early region of the genome of HCMV strain AD169 (Eco RI J fragment) and flank a 240bp fragment which appears to be well conserved in all isolates of HCMV investigated (Stanier et al, 1989). A nested primer CMN1, internal to CMP1 was used to confirm and amplify products obtained from PCR performed on PBMC DNA. The primer pair CMN1/CMP2 flank a 194bp fragment. A 17 mer oligonucleotide probe, CMPR1, was also synthesised, this is complementary to an internal region of the CMP1/CMP2 and CMN1/CMP2 products.

RNA PCR was performed using the 20 mer primers CMP3 and CMP4 (Table 2.1) located in the immediate early region of the genome of HCMV strain AD169, and which flank a 227bp fragment of the IE1 RNA from exon 4 (Sternberg et al, 1984). A 20 mer probe, CMPR2, was synthesised, this is complementary to an internal region of the CMP3/CMP4 product.
Table 2.1 Sequence of Oligonucleotide Primers and Probes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Oligo. size</th>
<th>Product size(bp)</th>
<th>Target nuc.acid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP1</td>
<td>CCCGACTTTACCATCCAGTA 20</td>
<td>240</td>
<td>DNA</td>
<td></td>
<td>Stanier et al 1989</td>
</tr>
<tr>
<td>CMP2</td>
<td>AAGACGAAGAGGAACTATCT 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMN1</td>
<td>GCTGTATCGTGATCTCTGAT 20</td>
<td>194*</td>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMPR1</td>
<td>GGGTGAAAGGAGTCGAAA 17</td>
<td></td>
<td>Probe</td>
<td>DNA</td>
<td>Stanier et al 1989</td>
</tr>
<tr>
<td>CMP3</td>
<td>CTATGCAGAGCATGTATGAG 20</td>
<td></td>
<td>RNA/(cDNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMP4</td>
<td>GGTCTTAGGAAGGCTGAGT 20</td>
<td></td>
<td></td>
<td>DNA</td>
<td></td>
</tr>
<tr>
<td>CMPR2</td>
<td>GAGATGTGGATGGCTTGAT 20</td>
<td></td>
<td>Probe</td>
<td>DNA</td>
<td></td>
</tr>
<tr>
<td>BEP1</td>
<td>ACACAACCTGTGTTCACTAGC 20</td>
<td>110</td>
<td>DNA</td>
<td></td>
<td>Saiki et al 1986</td>
</tr>
<tr>
<td>BEP2</td>
<td>CACTTCATCCACGTTCACC 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* when in the primer pair CMN1/CMP2

To monitor further the quality and quantity of the PBMC DNA prepared for PCR, a pair of human β-globin gene primers, BEP1 and BEP2 (Table 2.1), which flank a 110bp fragment, were used.

Oligonucleotides were obtained from a number of commercial and non-commercial sources, and were synthesised on DNA synthesisers: Applied Biosystems model 380, Pharmacia Gene Assembler Plus, and Biosearch Inc. Cyclone synthesiser. The oligonucleotides were obtained either vacuum dried and attached to their support columns, removed from the column and supplied as unpurified ethanol precipitates, or removed from the column and resuspended in distilled water. Where
necessary, solubilisation and deprotection were achieved by basic ester hydrolysis in ammonia, which was then removed by gel filtration using PD10 columns (Pharmacia). Fractions from the columns were collected, the A$_{260}$ measured and the relevant fractions pooled.

### 2.5.6.2 Analysis of Genomic and Viral DNA

The polymerase chain reaction (PCR) was performed essentially as described by Saiki 	extit{et al} (1985). Target DNA (0.5-3µg) was amplified in a reaction volume of 100µl, containing final concentrations of; 50mM KCl, 10mM Tris.HCl pH 8.4, 1.5mM MgCl$_2$, 100µg/ml gelatin, 200µM each dATP dCTP dGTP dTTP, 100pmol (0.66µg) of each primer and 1-2u AmpliTaq DNA polymerase (Perkin Elmer Cetus). All the reagents except the Taq polymerase were added, and the DNA denatured for 10 minutes at 94°C. The enzyme was added and the reaction mix overlaid with 100µl of mineral oil.

The standard reactions were performed using an automated thermal cycler (PHC-2, Techne), with cycle parameters of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C and elongation for 1 minute at 72°C. 35 cycles were performed, with the final cycle having an elongation time of 10 minutes to ensure completion of the final elongation step. Nested PCR was performed as above but with only 25 cycles.

After completion of the PCR, 10µl of the product was resolved on a 2% agarose/TBE gel containing 0.5µg/ml ethidium bromide. 2-3µg of 1kbp ladder (Gibco BRL) was loaded onto the gel as a DNA size marker. The gel was photographed using Polaroid type 47 or 667 film. For Southern
blotting, DNA was transferred onto Hybond N nylon membranes (Amersham) as described in section 2.5.7.

2.5.6.3 Analysis of Messenger RNA

PCR was performed on mRNA as described above for DNA, except that the PCR reaction was preceded by a reverse transcriptase (RT) reaction to produce a cDNA.RNA hybrid from the RNA template. The reverse transcriptase reaction was performed in a reaction volume of 20μl containing final concentrations of 50mM KCl, 10mM Tris.HCl pH 8.4, 1.5mM MgCl₂, 100μg/ml gelatin, 1mM each dNTP, 2μl Inhibit-Ace (5 Prime 3 Prime Inc), 100pmol (0.66μg) of the oligonucleotide primer complementary to the 3’ end of the mRNA target (CMP4), 1-10μg of total or in vitro transcribed RNA, and 200u of MuLV reverse transcriptase (Gibco-BRL). The reaction was incubated at room temperature for 10 minutes and then at 42°C for 45 minutes and then stopped by denaturing the enzyme at 95°C for 10 minutes and quick chilling on ice. DNA PCR was then performed by the addition PCR buffer, primer CMP3 and enzyme to a final volume of 100μl (with the final concentrations as detailed in section 2.5.6.2).

2.5.6.4 Prevention of Contamination

To minimise the risk of contamination of reagents and the consequent production of false positive PCR products (Kwock, 1989; Gibbs, 1989) the most stringent measures possible were taken. All manipulations were performed in a sterile cabinet, pipettes were cleaned regularly and reagents were checked for contamination on an individual basis. To minimise the number of operations, bulk reaction mixtures were prepared and aliquoted prior to the addition of sample and enzyme. The CMV.P1/P2 primers were restriction digested with Taq I restriction
endonuclease prior to use, the DNA fragment between the primers having a single, central Taq I restriction site.

2.5.7 Southern Blotting

Agarose DNA gels were transferred by capillary action onto either nitrocellulose (BA 85, Schleicher and Schuell) or nylon (Hybond N, Amersham) membranes according to the general method of Southern (1975). Gels were denatured by soaking twice for 30 minutes each in denaturing solution (Appendix) and then neutralised by soaking twice, for 30 minutes each in neutralising solution (Appendix). The DNA was transferred overnight onto the membrane using 2x SSC as the transfer buffer. The membranes were then rinsed in 2x SSC and then distilled water, air dried, and the DNA fixed by UV crosslinking. The filters were stored at room temperature under vacuum prior to hybridisation.

2.5.8 Northern Blotting

RNA was denatured in a solution containing final concentrations of: 50% formamide, 1x MOPS buffer (Appendix) and 16% formaldehyde (40% v/v), by heating at 65°C for 5 minutes. Denaturing agarose gels (1·5%) were prepared in 1x MOPS buffer containing 16% formaldehyde (40% v/v), and run in 1x MOPS buffer. The RNA was visualised by soaking the gel in 1x MOPS buffer containing 0·5μg/ml ethidium bromide for 15 minutes. The gel was destained by soaking in two changes of distilled water, for 30 minutes each.

The RNA was transferred by capillary action onto nylon membranes (Hybond N, Amersham) according to the method supplied by Amersham International (Membrane Transfer and Detection Methods).
Transfer was overnight using 6x SSPE as transfer buffer without any further denaturation. The membranes were rinsed in 6x SSPE and then distilled water, air dried and the RNA fixed by UV crosslinking. The filters were stored at room temperature under vacuum prior to hybridisation.

2.6 ANALYSIS OF DONOR SAMPLES

2.6.1 Serum Samples

Donor serum samples were analysed for the presence of HCMV DNA by dot-blot hybridisation and by PCR, and were screened for the presence of HCMV-IgG by EIA as described in section 2.2.1.

2.6.1.1 Dot Blot Analysis

A standard volume of serum (100µl) was treated with 2·2% NP40 and 0·67% 2-ME (final concentrations) for five minutes at room temperature to disrupt viral membranes. The sample was then placed on ice, an equal volume of of 2M NaCl added and the DNA denatured with two volumes of 1M NaOH. The denatured DNA was then dot-blotted onto nitrocellulose (BA 85, Schleicher & Schuell), prewetted with 6x SSC, using the Minifold II apparatus (Schleicher & Schuell), and the blots washed with neutralising solution (Appendix). The filter was then rinsed in 6x SSC and baked at 80°C, and subsequently probed using the ³²P labelled Eco RI J fragment of HCMV as described in section 2.5.3.1.
2.6.1.2 PCR Analysis

For PCR analysis, DNA was extracted from 100μl of serum by addition of SDS and digestion with pronase, followed by standard phenol/chloroform extraction and ethanol precipitation, and then resuspended in 10μl of distilled water and stored at -40°C. PCR was performed on the entire 10μl of extracted DNA using the primer pair CMP1/CMP2.

2.6.2 Peripheral Blood Mononuclear Cells (PBMC)

2.6.2.1 Separation of Donor PBMC

PBMCs were obtained from the 5ml anticoagulated blood samples by density gradient separation. The samples were well mixed, layered onto an equal volume of Ficoll/Triosil gradient and spun at 3000rpm for 20 minutes. The resulting band, containing predominately lymphocytes, was collected and the cells washed twice in PBS. These were transferred to Eppendorf tubes, resuspended in 1ml of STE (Appendix) and stored at -40°C.

2.6.2.2 Preparation of PBMC DNA for Dot-Blot Analysis

For dot-blot analysis, DNA was extracted from 100μl of cell suspension by addition of SDS and digestion with pronase, followed by standard phenol/chloroform extraction and ethanol precipitation and then resuspended in 100μl of TE pH 8.0 and stored at -40°C.

2.6.2.3 Dot-Blot Analysis

The DNA was denatured by boiling for ten minutes and quenching on ice. An equal volume of ice cold 20x SSC was added and the DNA immediately applied to nitrocellulose membrane as described in section
2.6.1.1. The membrane was probed using $^{32}$P labelled Eco RI J fragment of HCMV as described in section 2.5.3.1.

2.6.2.4 PCR Analysis of PBMC DNA

PBMC DNA was initially prepared for PCR analysis as described in section 2.6.2.2. The DNA was extracted from 500μl of cells, resuspended in 100μl of distilled water and stored at -40°C prior to PCR analysis. Subsequently, however, PBMCs similarly obtained by density gradient separation were lysed and used directly for PCR. Approximately $10^7$ cells were washed twice in PBS, transferred to Eppendorf tubes and resuspended in 500μl of PCR buffer containing 0.45% NP40 and 0.45% Tween 20, and 25μg/100μl of Proteinase K. They were digested at 56°C for 60 minutes and the digest then heated at 96°C for 15 minutes to inactivate the proteinase and any DNases present. The resulting lysate was spun briefly in a microfuge and then stored at -40°C prior to PCR analysis which was performed as previously described, using primer pair CMP1/CMP2. 10μl of extracted DNA or 25μl of cell lysate was used for each reaction, being equivalent to approximately 1μg of total genomic DNA.

2.6.2.5 PCR Analysis of PBMC RNA

Total RNA was isolated from PBMC using a minor modification of the method of Chomczynski & Sacchi (1983). Separated PBMC were resuspended in 1ml of STE and 500μl of this was pelleted and the cells lysed in 500μl of guanidinium thiocyanate denaturing solution (Appendix), followed sequentially by 50μl of 2M sodium acetate pH 4, 500μl of phenol and 100μl of chloroform–isoamyl alcohol (49:1), with thorough mixing between each addition. The final suspension was vortexed for 10-15 seconds and then placed on ice for 15 minutes. The
solution was spun at 13,000g for 20 minutes at 4°C and the
supernatant removed to a fresh tube. An equal volume of isopropanol
(IPA) was added and the RNA precipitated at -40°C for one hour. The
RNA was pelleted by spinning as above and the pellet redissolved in
300µl of the denaturing solution followed by the addition of 300µl of
IPA and precipitation at -40°C for one hour. The RNA was precipitated
once more, washed in 75% ethanol and finally resuspended in 20µl of
0·05% SDS in depc treated water. The RNA was finally digested with
10u of RNase free DNase for one hour at 37°C, heat inactivated at 98°C
for ten minutes and stored at -40°C prior to use. RNA PCR was
performed using 2-5µl of RNA in the RT reaction and using anti-sense
primer CMP4 as the RT template. DNA PCR was then performed on the
resulting ssDNA following addition of the sense primer CMP3.

2.6.3 urine Samples

2.6.3.1 Urine Collection

Urine samples were collected from apheresis donors and members of
staff who, at the time of sample collection, met the criteria for blood
donation. The urine samples were collected as early morning specimens
(total volume voided) as aseptically as possible. Three volumes of
urine (3x 2ml) were removed aseptically for virus culture, snap frozen
and stored in liquid nitrogen. The remainder was then clarified by
centrifugation at 3000rpm for 15 minutes at 15°C. 200ml was then
ultracentrifuged at 35K rpm for 1½ hours at 15°C to pellet virus
present. The pellet was suspended in 450µl of STE and stored at -40°C
prior to DNA extraction.
2.6.3.2 Urine Culture

HEL cells were grown to confluence using standard techniques in 10ml plastic culture tubes (Sterilin). Urine samples were processed as described above and 1ml of urine was diluted 1:2 with 2x maintenance medium containing a final concentration of 200µg/ml gentamicin. Medium was removed from the cells, and the diluted urine added. After incubation for 1-2 hours at 37°C the inoculum was carefully removed and 4ml of maintenance medium containing 200µg/ml gentamicin was added. The cells were incubated for a further two hours at 37°C, the medium removed, replaced with standard maintenance medium and incubated for 24 hours. The medium was then changed once more and the tube incubated for up to four weeks with changes of standard maintenance medium once or twice weekly. The cells were checked twice weekly for cpe. All urine samples were tested in duplicate and considered to be negative if no cpe was detected after four weeks of culture.

All samples demonstrating toxic effects and/or causing bacterial contamination of the cultures were re-cultured using the duplicate frozen stored sample. Samples which again gave similar results were considered unsuitable for culture.

2.6.3.3 DNA Extraction

The pelleted material was digested at 37°C for 3-4 hours following the addition of 25µl of 20mg/ml Pronase and 25µl of 10% SDS. The DNA was extracted as previously described, resuspended in 50µl of TE pH 8.0 and stored at -40°C.
2.6.3.4 Dot Blot Analysis

Initially, all 50μl of the extracted DNA was dot blotted onto nitrocellulose. Subsequently, when PCR analysis was also performed on these samples, only 40μl of the DNA preparation was used. The DNA was denatured by boiling for ten minutes and quenching on ice, an equal volume of ice cold 20x SSC added and the sample transferred to nitrocellulose as described in section 2.6.1.1. The membrane was probed using the $^{32}$P labelled Eco RI J fragment of HCMV as described in section 2.5.3.1.

2.6.3.5 PCR Analysis

For all samples processed prior to the instigation of PCR, analysis was performed retrospectively on DNA extracted from one of the 2ml samples of urine stored in liquid nitrogen. The urine sample was pelleted and the DNA extracted and then redissolved in 10μl of distilled water. For all samples processed following the instigation of PCR, the analysis was performed on 1/5th of the DNA (10μl) extracted from the pellet produced by ultracentrifugation of the urine (section 2.6.3.3). PCR analysis was performed on the urine DNA samples as previously described, using the primer pair CMP1/CMP2.

2.6.4 Saliva Samples

2.6.4.1 Saliva Collection.

Saliva samples were collected from the same group that provided the urine samples, and at the same time. The samples were filtered through a nylon mesh to remove any particulate matter, and then spun at 3000rpm for 10 minutes at RT. The clarified saliva was then made up to 40ml with PBS and any virus present pelleted at 30,000
rpm for 1½ hours at room temperature. The pellet was resuspended in 450μl STE and stored at -40°C prior to DNA extraction.

2.6.4.2 DNA Extraction

DNA was extracted from the ultracentrifuge pellet by standard extraction procedures, dissolved in 50μl of distilled water and stored at -40°C.

2.6.4.3 Dot-Blot Analysis

40μl of the extracted DNA was dot-blotted onto nitrocellulose membrane as described in section 2.6.1.1. The membrane was probed using the 32P-labelled Eco RI J fragment of HCMV.

2.6.4.4 PCR Analysis

PCR was performed on the remaining 10μl of extracted DNA using the primer pair CMP1/CMP2.
3.1 RESULTS

3.1.1 Prevalence of HCMV-Antibody

The serostatus of the donors was determined solely on the basis of presence or absence of HCMV-IgG. Serum samples from a total of 9027 normal blood donors were screened for the presence of HCMV-IgG. Of these, 4173 (46.2%) were seropositive and 4854 (53.8%) seronegative. The prevalence of HCMV-IgG was found to be proportional to donor age (Table 3.1, Figure 3.1), increasing with increasing age.

Table 3.1 Prevalence of HCMV-IgG related to donor age

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. tested</th>
<th>No. positive</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-20</td>
<td>382</td>
<td>80</td>
<td>20.9</td>
</tr>
<tr>
<td>21-25</td>
<td>1396</td>
<td>368</td>
<td>26.4</td>
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<tr>
<td>26-30</td>
<td>1467</td>
<td>525</td>
<td>35.8</td>
</tr>
<tr>
<td>31-35</td>
<td>1238</td>
<td>514</td>
<td>41.5</td>
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<tr>
<td>36-40</td>
<td>1241</td>
<td>618</td>
<td>49.8</td>
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<tr>
<td>41-45</td>
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<td>46-50</td>
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<td>425</td>
<td>289</td>
<td>68.0</td>
</tr>
<tr>
<td>61-65</td>
<td>173</td>
<td>127</td>
<td>73.4</td>
</tr>
<tr>
<td>Total</td>
<td>9027</td>
<td>4173</td>
<td>46.2</td>
</tr>
</tbody>
</table>
Figure 3.1  Histogram showing the prevalence of HCMV-IgG in blood donors related to age.
Table 3.2 Prevalence of HCMV-IgG in blood donors related to donor age and sex.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Male</th>
<th>Female</th>
<th>Male (%)</th>
<th>Female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-20</td>
<td>172</td>
<td>210</td>
<td>19.8</td>
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<td>21-25</td>
<td>670</td>
<td>726</td>
<td>24.3</td>
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<td>819</td>
<td>648</td>
<td>32.8</td>
<td>39.5</td>
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<tr>
<td>31-35</td>
<td>744</td>
<td>494</td>
<td>39.4</td>
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<tr>
<td>36-40</td>
<td>722</td>
<td>519</td>
<td>47.5</td>
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<td>729</td>
<td>467</td>
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<td>325</td>
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<td>77.9</td>
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<tr>
<td>TOTAL</td>
<td>5140</td>
<td>3887</td>
<td>44.4</td>
<td>48.6</td>
</tr>
</tbody>
</table>

The overall prevalence of HCMV-IgG in female donors (48.6%) was found to be significantly higher than that in male donors (44.4%) ($\chi^2 = 15.92, p < 0.001$) (Table 3.2). This higher prevalence was found in each age group, ranging from 2.1% in the 18-20 year age group, to a maximum of 13.8% in the 46-50 year age group.
Table 3.3 Prevalence of HCMV-IgG in UK blood donors

<table>
<thead>
<tr>
<th>Region/RTC</th>
<th>% Seropositive</th>
<th>Region/RTC</th>
<th>% Seropositive</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE Thames</td>
<td>46</td>
<td>NW Thames</td>
<td>48</td>
</tr>
<tr>
<td>Birmingham</td>
<td>50</td>
<td>SW Thames</td>
<td>47</td>
</tr>
<tr>
<td>Bristol</td>
<td>40</td>
<td>Sheffield</td>
<td>45</td>
</tr>
<tr>
<td>Cardiff</td>
<td>45</td>
<td>Newcastle</td>
<td>45</td>
</tr>
<tr>
<td>Liverpool</td>
<td>50</td>
<td>Manchester</td>
<td>45</td>
</tr>
</tbody>
</table>

Mean prevalence 46.1%

3.1.2 Prevalence of HCMV-IgG in Blood donors in England and Wales

The prevalence of HCMV-IgG in blood donors in other health regions of the UK is presented in Table 3.3. These figures were correct in December 1990, and represent the results of the routine testing of donations for HCMV-IgG, though a number of different commercial HCMV-Ab assays were used.

3.1.3 Seroconversion

A group of 836 seronegative donors were studied over a period of approximately three years in order to determine the actual seroconversion rate in that population. Although prospective in design, the study was actually performed on retrospective data from the results of the routine screening of donations for HCMV-IgG. The 836 donors were identified as having been found to be HCMV-IgG negative during the period between 01/01/87 and 30/06/88, and then having made subsequent donations over a minimum period of one year and having been rescreened for the presence of HCMV-IgG.
Table 3.4 Observed and expected seroconversion rates

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. studied</th>
<th>Seroconv. obs.</th>
<th>Seroconv. exp.</th>
<th>Mean seroconv. period (yr)</th>
<th>Annual seroconv. rate(%)</th>
<th>obs.</th>
<th>exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-20</td>
<td>43</td>
<td>2</td>
<td>3</td>
<td>1.1</td>
<td>1.6</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>21-25</td>
<td>163</td>
<td>10</td>
<td>13</td>
<td>1.0</td>
<td>2.1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>26-30</td>
<td>190</td>
<td>8</td>
<td>11</td>
<td>0.8</td>
<td>1.4</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>31-35</td>
<td>115</td>
<td>8</td>
<td>10</td>
<td>1.4</td>
<td>2.3</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>36-40</td>
<td>110</td>
<td>5</td>
<td>9</td>
<td>0.9</td>
<td>1.5</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>41-45</td>
<td>100</td>
<td>3</td>
<td>8</td>
<td>1.1</td>
<td>1.0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>46-50</td>
<td>58</td>
<td>3</td>
<td>7</td>
<td>1.3</td>
<td>1.7</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>51-55</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>56-60</td>
<td>20</td>
<td>0</td>
<td>2</td>
<td>-</td>
<td>0</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>61-65</td>
<td>6</td>
<td>0</td>
<td>n/k</td>
<td>-</td>
<td>0</td>
<td>n/k</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>836</td>
<td>39</td>
<td>63</td>
<td>Mean 1.09</td>
<td>1.66</td>
<td>2.53</td>
<td></td>
</tr>
</tbody>
</table>

n/k = Not Known (donors are only bled up to the age of 65)
obs = observed      exp = expected

1 negative value obtained because of the decrease in prevalence of HCMV-specific Ab seen in the 56-60 year age group as compared to the 51-55 year age group

The observed and expected seroconversion rates of seronegative donors during the study period are presented in Table 3.4. The observed seroconversion rate was calculated from the total number of seroconversions that were identified during the three year study period. For ease of calculation it was assumed that seroconversions were spread evenly throughout the three year study period. The rate was expressed as the mean rate of seroconversion.
per year in the donor population, and further expressed as the mean rate of seroconversion within each age group.

The expected annual seroconversion rate for each age group was calculated, on a cumulative basis, from the theoretical number of seroconversions in the normal donor population that needed to occur to account for the increase in the prevalence of HCMV-IgG observed from one age group to the next. The rate is expressed as the expected mean annual seroconversion rate within each group.

The figures for the expected number of seroconverting donors were calculated by applying the expected seroconversion rates for the age group to the total number of seronegative donors studied in that age group, and expressing the results as the total number of seroconversions expected to occur during the three year study period in this study population. This calculation was repeated for each age group. In all cases the observed seroconversion rate was less than the expected rate. Although a mean seroconversion period was calculated, this figure relates more to the frequency of donation rather than any accurate assessment of the timing of infection and subsequent seroconversion in the donor population.
Overall, 4173 of 9027 (46.2%) blood donors in the North East Thames Region were found to possess HCMV-IgG, which is in good agreement with the figure of 48% recently reported by McDonald et al. (1990) for blood donors in the N.W.Thames Region, a similar donor population. Further, unreported data from other UK Regional Transfusion Centres (Table 3.3) supports these figures from the N.W. and N.E.Thames regions and demonstrates a fairly uniform prevalence across the UK. This differs significantly from the wide ranging levels reported for other countries, for example 20-70% in the USA (Tegtmeier, 1986). Recently, however, Klapper et al. (1990) have reported a rather higher prevalence of HCMV-IgG in blood donors from the Manchester RTC, using a new radioimmunosorbent test (RIST). They reported a prevalence of HCMV-IgG of 53% in the donors tested. This figure, however, is higher than that of 45% reported by Manchester RTC itself (Table 3.3) from data determined using a commercial HCMV-Ab assay. If the use of this new assay provides a genuine increase in sensitivity, such a large increase in the estimated prevalence of HCMV-IgG positive individuals may be general throughout the UK and requires further study. However, the assays currently used by the RTC’s are all standard and also used in clinical laboratories without any reported sensitivity problems. It is quite possible that the donor samples provided for testing using the RIST assay may have been collected at a donor session in an area with a localised higher prevalence of HCMV-IgG. Alternatively, the specificity of the assay may need further examination.

The presence of HCMV-Ab is clearly linked to age. The prevalence in the lowest age group (18-20 years) being 20.9% and rising to 73.4% in
the 60-65 age group. Interestingly, there is a significant decrease in the prevalence of antibody in the 56-60 age group, a finding similar to that of Eley (1987) in an earlier, smaller study of donors from the N.E. Thames region. McDonald et al (1990) studied the prevalence of HCMV-IgG in four age groups of plasmapheresis donors, and showed a similar increase in prevalence with age. They also noticed a decrease in prevalence in the oldest age group (50-59 years). The reasons for this decrease in prevalence are unclear but, as this has been seen in three different studies, it is unlikely to be attributable to bias in the study population. A possible explanation for the finding is that the decrease is specifically associated with those donors currently in that age group, and this decrease has always been associated with those donors in previous years. Thus it may represent the result of an earlier event resulting in a localised decrease in the HCMV infection rate, and hence HCMV-IgG prevalence, in those donors.

The finding of a significantly higher prevalence of HCMV-IgG in female donors is a further puzzling finding. The reasons for this difference are unclear but a similar difference was found by Wilhelm et al (1986) who noted an even greater discrepancy. The prevalence of HCMV-IgG was found to be 45% in female donors but only 36% in male donors. No reasons for this difference were put forward by these authors but they did make the further significant comment that the overall rate of infection, marked by seroconversion, in female patients was correspondingly higher than that in male patients. It is known that pregnancy often leads to reactivation of infection or increased risk of primary infection. However, this is unlikely to be the sole reason for such a finding, as the difference is clearly seen in the younger age groups, the majority of whom are unlikely to have been pregnant.
Furthermore, only primary infection would produce an increase in the prevalence of HCMV-Ab, reinfection or recurrent infection being undetectable on the basis of HCMV-IgG screening alone. Although the complex relationship between HCMV and the immune system provides potential for increased susceptibility to infection (c.f. HLA and HCMV infection) no known links between HLA and gender have been established. Nevertheless, it is hard to envisage any reasonable mechanism by which natural HCMV infection would preferentially infect healthy females without postulating either a greater protection afforded by the male immune system or a greater female susceptibility to HCMV infection.

The current seroconversion rate in UK blood donors is generally accepted as 1-2% per annum (Entwhistle & Tobin, 1984). Although the overall seroconversion rate observed agrees with this estimate, the figures do not accord with the calculated expected seroconversion rate. If it is assumed that the increasing prevalence of HCMV-IgG seen in donors is due to HCMV infection occurring both naturally and constantly throughout the general population and accounts for the increase in prevalence of HCMV-Ab from one age group to the next, the seroconversion rate for any one age group must equate with the increase in prevalence seen in the next age group. On this basis the overall expected seroconversion rate was calculated as was that for each age group. Surprisingly, however, these rates are significantly higher than the 1-2% observed seroconversion rate, and further require that the incidence of primary infection specifically increases with increasing donor age.

It is noticeable that the highest discrepancy between the rates is found in the older age groups, where no incidences of seroconversion
were seen. However, this may partly be due to the sample size, as the total number of older donors included in the study was relatively low. The period with the highest frequency of natural infection in immunocompetent individuals in developed countries is generally considered to be adolescence and early adulthood. However, the seroconversion rates seen in the youngest donor age groups were no greater than those seen in other age groups; those not generally considered to be associated with a high incidence of infection. It would appear that the current seroconversion rate is essentially constant, at least between the ages of 18 to 50.

If, however, the assumption made above is not correct or only partially correct, the calculations subsequently performed to obtain the expected seroconversion rates may not be valid. If this is the case, the age related prevalence of HCMV-IgG in the study population may not simply reflect seroconversion from one age group to the next, but may reflect previous higher rates of infection, probably during late childhood and early adolescence. If, as suspected, the prevalence of virus and hence the incidence of primary infection is slowly declining in immunocompetent individuals with the development of society, the higher prevalence of HCMV-Ab in the older age groups may reflect not just current infection and subsequent seroconversion, but significantly, a much higher incidence of infection in earlier life. Those donors currently in the older age groups would therefore have had a higher prevalence of HCMV-Ab when aged 21-25, for example, than the prevalence in the donors currently in that age group.

Although very few studies of the prevalence of HCMV-IgG in blood donors in the UK have been published, there are some, albeit limited, data to support this view. The overall prevalence of HCMV-IgG in a
similar donor population in 1971 was found to be 55% (Perham et al., 1971). Continued improvements are being made to the general hygiene and living conditions in many areas of the world, including the UK, two factors known to play an important role in the incidence of transmission of HCMV in immunocompetent individuals. This improvement in conditions has recently been highlighted, for example, in the decreasing prevalence of Ab to Hepatitis A Virus (HAV-Ab) in blood donors.

The increase in the number of ethnic individuals in the general population may be another factor affecting the prevalence of HCMV-Ab. Such individuals who were born outside of this country in countries with poorer living conditions and a higher incidence of HCMV infection would have encountered HCMV much earlier in their lives than similar individuals in western countries. The extent of any such increase is, however, unknown. Although donor sessions in the N.E.Thames region are held in areas of ethnic populations, the proportion of ethnic donors is not representative of the numbers in the overall population of these areas. The impact of any such donors on general donor trends is, therefore, currently unknown and difficult to assess accurately.

The possibility of random, minor, localised epidemics of HCMV cannot be dismissed. Transmission in immunocompetent individuals represents the major route of infection by HCMV, and provides a continual pool of infectious individuals, but it is clear that acute symptomatic infection is usually marked only by mild clinical symptoms and many cases of acute infection are asymptomatic. It is likely, for example, that some of the so-called 'flu' outbreaks are not due specifically to influenza viruses, but are due to one or more of the many other respiratory
tract viruses, including HCMV, infection with which can give rise to 'cold- or flu-like symptoms' of varying severity. It is possible that HCMV may feature regularly among those viruses actually causing these 'flu' outbreaks.
CHAPTER 4 QUANTITATIVE AND QUALITATIVE ASPECTS OF THE IMMUNE RESPONSE TO HCMV

4.1 RESULTS

4.1.1 Titre of HCMV-IgG in Blood Donors

Donors were divided into three groups on the basis of HCMV-IgG titre. These groups were defined as: low titre - 1/1 to 1/8, medium titre - 1/16 to 1/64 and high titre - > 1/64. The HCMV-IgG titres of 500 donors were determined using the 'in house' HCMV-IgG assay. Correlation between the HCMV-IgG titre and donor age was investigated and the results are presented in Table 4.1.

Table 4.1 HCMV-IgG Titre Related to Donor Age

<table>
<thead>
<tr>
<th>Age group</th>
<th>Male donors</th>
<th></th>
<th>Female donors</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean titre</td>
<td>Range</td>
<td>No. of donors</td>
<td>Mean titre</td>
</tr>
<tr>
<td>18-20</td>
<td>26</td>
<td>2-128</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>21-25</td>
<td>23</td>
<td>4-32</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>26-30</td>
<td>16</td>
<td>2-64</td>
<td>31</td>
<td>16</td>
</tr>
<tr>
<td>31-35</td>
<td>34</td>
<td>2-128</td>
<td>47</td>
<td>22</td>
</tr>
<tr>
<td>36-40</td>
<td>19</td>
<td>1-128</td>
<td>53</td>
<td>22</td>
</tr>
<tr>
<td>41-45</td>
<td>34</td>
<td>2-128</td>
<td>48</td>
<td>49</td>
</tr>
<tr>
<td>46-50</td>
<td>30</td>
<td>1-64</td>
<td>33</td>
<td>17</td>
</tr>
<tr>
<td>51-55</td>
<td>33</td>
<td>2-128</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td>56-60</td>
<td>20</td>
<td>2-64</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>61-65</td>
<td>15</td>
<td>2-32</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>23.9</strong></td>
<td>-</td>
<td><strong>275</strong></td>
<td><strong>29</strong></td>
</tr>
</tbody>
</table>
Of the 500 donors, 332 (66.4%) were found to be low titre, 127 (25.4%) medium titre, and 41 (8.2%) high titre. No correlation was demonstrated between the age of the donor and the antibody titre ($t = 0.301$, $p < 0.05$) nor was a significant difference observed between the titres found in male and female donors ($\chi^2 = 9.42$, $p < 0.05$).

4.1.2 Immunoblotting Studies on HCMV-IgG Positive Donors

The sera for study were obtained from normal blood donors, although some clinical sera were also examined. The sera from blood donors were grouped according to titre, using the ranges defined in Section 4.1.1. Immunoblotting was performed in order to detect the HCMV-specific structural polypeptides recognised by IgG antibodies. Some variation in the positions of some of the peptides was apparent, this was due to some differences in the SDS-PAGE conditions.

The general IB pattern of HCMV-IgG positive sera is shown in Plate 4.1. In total, 22 different polypeptides were recognised, with apparent MWs of 212, 150, 145, 125, 115, 97, 88, 84, 78, 74, 68, 64, 60, 55, 48, 45, 40, 38, 35, 33, 30, and 28 kDa. Both quantitative and qualitative differences were apparent between the responses in different individuals (Plate 4.1, Figure 4.1). Sera containing high titre HCMV-IgG usually gave more intense blotting patterns, although specific differences in the polypeptides recognised were also seen in donors with different titres of HCMV-IgG. Polypeptides of apparent MWs of 150, 97, 55, and 28 kDa appeared to be the most immunogenic as they generally gave strong signals and were recognised by the highest percentage of sera (Figure 4.1). One of these polypeptides, 150 kDa, was recognised by all sera, polypeptides 97, 55 and 28 kDa were
Plate 4.1  IB profile of HCMV-IgG positive donor sera, AD169 as blotting antigen. Samples 1-13 are from -IgG positive sera, sample 14 is -IgG negative. A non-specific band (58-60 kDa) can be seen on strip 14. Numbers on the left hand side indicate approximate MWs (kDa).
Figure 4.1 Percentage reactivity of individual HCMV polypeptides with HCMV-IgG present in human sera grouped according to Ab titre.

*ht* - high titre, *mt* - medium titre, *lt* - low titre, numbers along the bottom represent approximate MWs of the polypeptides.
recognised by 89·2, 97·2 and 84·1% of sera respectively. None of the three groups of sera entirely failed to recognise any of the specific polypeptides identified, at least one serum in each group identifying any particular polypeptide.

4.1.3 Effect of Strain Variation on Antigens used for Immunoblotting

Antigen for IB was also prepared from the HCMV laboratory strains Towne and Davis, and from six low passage clinical isolates. Strips prepared using these antigens were compared for polypeptides recognised by a serum containing HCMV-IgG. Some qualitative differences are seen in the IB patterns using different antigens (Plate 4.2). All strains studied produced the same basic IB profile routinely produced using strain AD169 as blotting antigen, containing polypeptides of identical MWs. Variation between strains was limited and appeared to result in the variable production of polypeptides of apparent MWs of 125, 84, 74, 48, 45, 40, 38, 35, 33 kDa by the different virus strains (Plate 4.2). There was also variation in the intensity of the reactions seen with some of the polypeptides.

4.1.4 Cross-reactivity of HCMV-Proteins With Specific Antibody to Other Herpesviruses

Sera containing high titre Abs to other herpesviruses (HSV I, -II, VZ, EB, HHV6) but negative for HCMV-Ab were examined by IB against AD169 antigen. No specific bands were seen with any of the sera.

4.1.5 Stability of the Immune Response to HCMV

To examine the natural variation in titre in normal individuals, a total of 60 random donors were studied retrospectively covering a
Plate 4.2  Variation in the IB profile of HCMV-IgG positive donor serum seen when different strains of HCMV are used as blotting antigen. Antigens used: strip 1- AD169, 2- Towne, 3- Davis, 4-9 clinical isolates. Results represent the profiles obtained when a single HCMV-IgG positive serum was tested against all 9 antigens. Numbers on the LH side indicate approximate MWs (kDa).
Table 4.2 Temporal Variation in Titre of HCMV-IgG in Blood Donors

<table>
<thead>
<tr>
<th>Initial titre</th>
<th>Number</th>
<th>No. inc. in titre</th>
<th>No. dec. in titre</th>
<th>Mean no. samples</th>
<th>Mean study period (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>9</td>
<td>0</td>
<td>1(^1)</td>
<td>3.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Medium</td>
<td>23</td>
<td>2(^2)</td>
<td>1(^3)</td>
<td>3.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Low</td>
<td>28</td>
<td>3(^4)</td>
<td>0</td>
<td>3.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>5</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) donor decreased in titre 1/256 to 1/32
\(^2\) 1 donor increased in titre 1/16 to 1/64, 1 donor increased in titre 1/64 to 1/256
\(^3\) donor decreased in titre 1/16 to 1/4
\(^4\) 1 donor increased in titre 1/4 to 1/16, 1 donor increased in titre 1/2 to 1/8, 1 donor increased in titre 1/8 to 1/32

minimum period of 18 months and a minimum of 3 donations per donor. Only variations in titre of more than one dilution from the initial titre were considered to be a change in titre. 54 of the 60 donors (88.3%) maintained their titre, 5 (8.3%) increased in titre, and 2 (3.3%) decreased in titre (Table 4.2).

Similarly, the HCMV-IgG titres of 20 plasmapheresis donors, plasmapheresed for a number of different high titre antibodies, were monitored for a period of at least one year to examine the effect of the frequent removal of defined volumes of plasma (≈500ml) on antibody titre (Table 4.3). 17 of the 20 (85%) maintained their titre, 1 (5%) increased in titre, and 2 (10%) decreased in titre.
Table 4.3 Temporal Variation in Titre of HCMV-IgG in Plasmapheresis donors

<table>
<thead>
<tr>
<th>Initial titre</th>
<th>Total No.</th>
<th>Inc. No. in titre</th>
<th>Dec. No. in titre</th>
<th>Mean no.</th>
<th>Mean study period (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>1.6</td>
</tr>
<tr>
<td>Medium</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td>Low</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>1</td>
<td>2</td>
<td>5.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

1 donor increased in titre 1/16 to 1/64
2 donor decreased in titre 1/32 to 1/8
3 donor decreased in titre 1/8 to 1/2

4.1.6 Temporal Variation in HCMV-IgG Immunoblotting Profile

Plate 4.3 shows the IB profile obtained from serum samples collected from three donors over a period of approximately 2 years. Two of the donors (1 and 2) demonstrated a decrease in HCMV-IgG titre (Table 4.2 donors 1 and 3), and donor 3 was a plasmapheresis donor who maintained HCMV-IgG titre. No significant changes were seen in the IB profiles of donor 3. The IB profile of donor 1 did show a decrease in the number of polypeptides detected. The decrease in titre in this donor corresponded to a loss in the detection of polypeptides with apparent MWs of 115, 68, 45, 40, 38 kDa and a decrease in intensity of reactions with polypeptides with apparent MWs of 125, 55 and 28 kDa.

4.1.7 Use of IB to Determine True HCMV-IgG Status

A number of serum samples from blood donors from other RTCs were referred for IB on the basis of discrepant results between commercial assays, both between different EIAs and between EIA and latex
Plate 4.3 Temporal variation in HCMV-IgG IB profile. Donors 1 and 2 are normal donors, donor 3 is a plasmapheresis donor. The IB profiles of donors 2 and 3 do not change over the study period. With donor 1, the response against polypeptides of MWs 125, 115, 68, 45, 40 and 38 kDa became undetectable, whilst responses against other polypeptides decreased in intensity. AD169 was used as blotting antigen. Numbers on the LH side indicate approximate molecular weights (kDa). Numbers along the top indicate the time period (months) since the first monitored donation (0).
Plate 4.4  HCMV-IgG IB profiles of weak/non-specific reacting donor sera. Samples 1-10 undetermined sera as detailed in Table 4.5, 11- positive serum, 12- negative serum. AD169 was used as blotting antigen. Numbers on the LH side indicate approximate molecular weights (kDa).
Table 4.4  HCMV-IgG IB Profile in Weakly/Non Specific Reactive Donor Samples

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Routine screening results</th>
<th>Polypeptides recognised(kDa)</th>
<th>Presumed HCMV serostatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EIA-1 neg/EIA-2 pos</td>
<td>150</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>2</td>
<td>IHA pos/EIA neg</td>
<td>-</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>3</td>
<td>IHA pos/EIA neg</td>
<td>58/60</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>4</td>
<td>Latex pos/EIA neg</td>
<td>-</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>5</td>
<td>Latex pos/EIA-1 pos/EIA-2 neg</td>
<td>150,88,64,55</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>6</td>
<td>EIA-1 pos/EIA-2 neg</td>
<td>58/60</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>7</td>
<td>IHA pos/EIA neg</td>
<td>-</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>8</td>
<td>EIA-1 neg/EIA-2 pos</td>
<td>58/60</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>9</td>
<td>Latex neg/EIA pos</td>
<td>150,125,97,68,64,55</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>10</td>
<td>IHA pos/EIA neg</td>
<td>-</td>
<td>NEGATIVE</td>
</tr>
</tbody>
</table>

particle (CMV Scan, Becton Dickinson) or indirect haemagglutination (IH) (Cetus) assays. Samples were tested blind and the routine screening results subsequently provided. Table 4.4 details the original and IB results on these samples. The corresponding IB profiles are shown in Plate 4.4.

The presence of the 150 and/or 55 kDa polypeptide bands was considered to mark the presence of specific HCMV-IgG in these sera, and to be suitable as a marker of specific HCMV-Ab in all such sera. The presence of other bands, not corresponding to previously recognised HCMV-specific proteins, was considered to be due to non-specific antibodies recognising cellular proteins. Indeed, on a number of occasions some non-specific bands were seen on the negative control strips run with the individual IB assays (Plate 4.1, lane 14).
4.2 DISCUSSION

An important factor in the current screening regimens, which are based upon the detection of HCMV-IgG to identify potential infectivity in donations, is the stability of the Ab response to HCMV. This question of stability has been considered by a number of authors with varying results. In this study, HCMV-IgG levels were found to be maintained in 56 of 60 (92%) normal donors whose donations were studied over a mean period of 2.3 years, and 18 of 20 (90%) plasmapheresis donors whose donations were studied over a mean period of 15 months. A high degree of stability in HCMV-IgG titre was, therefore, demonstrated in these donors. Waner et al (1973) studied HCMV-IgG levels in plasmapheresis donors and noted large fluctuations in titre in some individuals over an 18 month period. However, the study was based upon the use of CF to detect HCMV-IgG. The lower sensitivity of this technique may account for the detection of minor fluctuations in titre which do not affect a more sensitive assay such as EIA, and are consequently not found when an EIA is used. The frequency of donation was also high, with an average of 40 per year.

The results of MacDonald et al (1990) give support to the findings reported in this thesis. They found HCMV-Ab titre to be stable in donors; this stability has facilitated the maintenance of a panel of plasmapheresis donors possessing high titre HCMV-Ab suitable for HT-Ig production.

Fluctuations in HCMV-IgG titre have been demonstrated later in life, when age-related reactivation of HCMV has been postulated (Musiani et al, 1988; McVoy & Adler, 1989). Significant increases in titre of HCMV-IgG and appearance of HCMV-IgM have been found in elderly individuals (> 70 years). Both studies postulated that the increases
resulted from reactivation of virus, not from primary infection, and this was as a result of decreased cell mediated immunity associated with aging.

However, the stability of the Ab response is perhaps more critical in the routine screening of donors and the provision of HCMV-Ab negative blood and products. The effective absence of HCMV-Ab due to a decline to below detectable levels may have two important implications. Firstly a donation may be considered to be HCMV-Ab negative and hence present a real risk of HCMV transmission if transfused to an HCMV-Ab negative recipient. Secondly, a potential recipient may be screened as HCMV-Ab negative and therefore be supported with HCMV negative products unnecessarily, this may also result in undue delay if suitable products are not readily available.

The reasons for these fluctuations of titre in healthy donors are unclear. Reinfection with another strain of HCMV could be expected to induce a rise in HCMV-IgG titre in a small number of donors. Decrease in titre may simply reflect a gradual decrease in HCMV-IgG production following primary infection and without any further stimulation by secondary infections. However, the presence of latent virus in these individuals would potentially provide a continual stimulus to the maintenance of stable HCMV-IgG levels. It is, therefore, possible that the decrease in HCMV-IgG titre found in a small number of donors represents individuals in whom latency either did not establish or established at lower virus levels than normal. A test of this supposition would be DNA PCR analysis of the PBMC from those donors demonstrating a decrease in HCMV-IgG titre. Examination of the DNA PCR data obtained during the course of this study (Chapter 7) does

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indeed establish that a number of HCMV-IgG positive donors do not possess detectable HCMV nucleic acid in their PBMC.

The IB results obtained in this study show a remarkable consistency in the profiles obtained from the analysis of HCMV-IgG sera. Unfortunately, the variety of IB methods used by different authors makes direct comparison of results difficult. It is clear from the review of Landini & Michelson (1988) that many authors have identified the same polypeptides although the putative MWs assigned to them by each author may be different. Differences in gel preparation, electrophoresis methods, transfer procedures and source of molecular weight markers together contribute to variability in the relative positions of identical polypeptides and hence the assignment of different MWs.

The use of infected cell extracts as blotting antigens in this study is an aspect that warrants further consideration. The source of the antigen, purified virions or infected cell extract, may affect the results obtained and has been the subject of some discussion. In the case of HCMV, there is no real consensus on this point, although some authors advocate the use of purified virions to reduce any reactivity due to cellular proteins. However, the relationship between virus-specific proteins, virus-induced proteins and cellular proteins can be complex and confusing. Two important findings have been made involving virus and host cell proteins; some of the structural proteins encoded by the virus are only detected in infected cells and not in virion preparations and host cell proteins have been identified in purified virion preparations, not as adhering contaminants but as integral virion proteins, (Landini & Michelson, 1988). In view of these findings, it was thought that infected cell lysates were the best
source of antigen for HCMV IB studies. A further important and more practical consideration is that cell lysates are generally easier to produce than purified virion preparations, although strict standardisation of cell culture procedures is required.

It is interesting to speculate as to whether differences in the antigen source due to different preparation methods, although from the same strain of virus, account for the variability in MWs assigned to the same polypeptide by the numerous reported studies (Landini & Michelson, 1988). Because of the tremendous variation in the reported MWs of the polypeptides recognised by the immune response to HCMV, a standard blotting profile has not been described. However, there is general agreement concerning the identity of some polypeptides, including those with apparent MWs of 150, 55, 38, and 28 kDa, interestingly, those eliciting some of the strongest and most consistent Ab responses. The polypeptides recognised in this study all conform with those previously recognised and reported (Landini & Michelson, 1988).

It is not clear whether the data presented in Figure 4.1 demonstrate quantitative or qualitative differences, or both, in the IB profiles of different individuals. The general pattern of the frequency of detection of the individual polypeptides can be seen to be maintained in all three groups of HCMV-IgG positive donors. The frequency of recognition of most, but not all, of the polypeptides decreases with decreasing titre of HCMV-IgG. Some of the weaker reacting polypeptides, either due to smaller amounts of polypeptide expressed in the infected cell or to low titre or low avidity polypeptide-specific Ab, showed quantitative differences, only being detected by higher titre sera - 130, 88, 68, 38, 35 kDa; others were recognised by
approximately the same proportion of sera from all three titre groups - 150, 97, 64, 55, 45, 28 kDa, thereby demonstrating a more qualitative difference.

In view of the large number of proteins produced by HCMV it is rather surprising that no cross-reactivity was seen between HCMV proteins and Abs raised against other herpesviruses. This finding does, however, validate the specificity of the IB technique. Some authors have detected specific areas of apparent NA homology between members of the herpesvirus family with a view to demonstrating regions of related sequence in the genome and the subsequent production of related proteins. Whilst some of the proteins of HCMV have been mapped to specific areas of the genome, significant sequence homology with other herpesviruses has not been found.

Both the finding of a common IB pattern in sera from seropositive individuals (Plate 4.1), and the demonstration of similar blotting patterns from a single serum with different strains of HCMV as antigen (Plate 4.2), provide evidence that antigenic variation, although present, is not a significant factor in the detection of HCMV-Ab in human sera. It seems reasonable to surmise that sufficient immunogenic structural proteins are conserved amongst most strains of HCMV to elicit similar immune responses from infected individuals, thus facilitating detection of HCMV-Ab in those individuals through the use of a single strain of antigen. Most commercial EIAs use the laboratory strain of HCMV, AD169, as their antigen source. Whilst most currently available commercial HCMV-Ab assays do show good agreement between their results, more detailed analysis demonstrates variation in the specificity rather than sensitivity of the assays (Kitchen, unpublished data), this being the cause of many of the discrepancies between
otherwise comparable assays. Specificity problems invariably arise when efforts are made to enhance the reactivity of assays to improve sensitivity. With a number of screening assays, for example to detect HIV-Ab, specificity problems are resolved simply by repeat and, if necessary, confirmatory testing. This rationale, however, is dependant on the low frequency of positive sera, enabling the repeat testing of only initially positive samples, and the availability of suitable confirmatory tests. Neither are appropriate in the case of HCMV-Ab screening. The repeat testing of more than 40% of donors tested is not feasible, and suitable confirmatory assays for HCMV-Ab are not available.

Both antigenic heterogeneity and variation in restriction enzyme profiles have been recognised in HCMV for a number of years though it seems that the effects of antigenic heterogeneity are more noticeable when using CF and indirect haemagglutination techniques (Waner & Welder, 1978; Faix, 1985). Faix (1985) tested random cord sera for HCMV-IgG by CF and IHA using three different antigens. He concluded that the use of a single antigen could lead to the incorrect classification of sera as HCMV-seronegative in as many as 6% of samples tested. Subsequently, Adler & McVoy (1986) used 10 different strains of HCMV as antigens to screen 259 blood donor samples for the presence of HCMV-IgG by EIA. They found no discrepancies in their study, all the HCMV-IgG positive sera were detected by all 10 antigens.

Recently, however, antigenic heterogeneity has been demonstrated using MoAbs to detect the differences between strains (James & Cloonan, 1990). MoAbs have specific advantages in the study of antigenic heterogeneity as they can reveal subtle differences between
strains of virus that polyclonal Abs may not detect. However, whilst this heterogeneity may have some relevance in tests using MoAbs for the rapid detection of virus in clinical infections, these findings do not invalidate the use of a single antigen specificity in routine assays for the detection of natural HCMV-Ab.

Sufficient variation in HCMV DNA exists to allow discrimination between any two strains by restriction endonuclease analysis, though only a single nucleotide change is required to alter a restriction site, and this alteration may have little or no effect on the encoded protein.

Although it is possible that antigenic heterogeneity could be an advantage to the virus, potentially enabling infection of a previously infected individual by different strains of virus, this is unlikely to be the case. Because HCMV-specific Ab appears to be non-neutralising, virus and specific antibody can be simultaneously isolated from the same sites, the protective effect of HCMV-Ab is unclear. Thus antigenic heterogeneity cannot be considered as a means of avoiding the host humoral immune response.
CHAPTER 5 HYBRIDISATION ANALYSIS

5.1 RESULTS

5.1.1 Dot-Blot Analysis

5.1.1.1 Specificity

No hybridisation was detected between the *Eco* RI J fragment probes and DNA from the other human herpesviruses (HSV I, -II, EB, VZ, HHV-6), uninfected HEL DNA or PBMC DNA from HCMV-IgG negative donors.

5.1.1.2 Sensitivity

Radiolabelled ($^{32}$P) *Eco* RI J fragment probes were routinely produced with specific activities of $10^6$-$10^8$ cpm/µg. Using these probes, 0.5-2 pg of plasmid pCM5018 and 10-20 pg of purified HCMV-DNA could be detected routinely in reconstruction experiments. No difference in sensitivity was observed following similar reconstruction experiments with pCM5018 in the presence of 2-3µg of uninfected HEL DNA (Plate 5.1).

1pg of plasmid pCM5018 is equivalent to approximately 62,000 copies of the cloned fragment and 10pg of HCMV DNA approximately 38,000 copies of the HCMV genome. Detection of 1pg of plasmid DNA and 10pg of HCMV DNA therefore is approximately equivalent in terms of the number of copies of that region of the virus genome detected.

The probe hybridised with DNA extracted from all of the clinical isolates of HCMV obtained during the course of this study.
Plate 5.1  Sensitivity of HCMV dot-blot analysis. Dot-blot analysis of known quantities - 50, 20, 10, 5, 3, 2, 1 and 0.5 pg of HCMV DNA. A - pCM5018 plus 2–3 µg of HEL DNA, B - pCM5018 alone, C - HCMV (AD169) DNA, probed with ³²P labelled *Eco RI J* fragment of HCMV.
5.1.2 Detection of HCMV DNA by PCR

5.1.2.1 Specificity

No specific amplification products were obtained using either of the HCMV-specific primer pairs (CMP1/CMP2 and CMP3/CMP4) when DNA PCR was performed on DNA extracted from cultured cells infected with the other human herpesviruses (HSV I, -II, EB, VZV, HHV-6), or with uninfected HEL cell DNA.

5.1.2.2 Sensitivity

Using the primer pair CMP1/CMP2 to amplify titrated plasmid pCM5018, the specific amplification product was detected on a 2% agarose/EtBr gel from 1fg of plasmid DNA. This sensitivity was confirmed by Southern blotting and hybridisation using a $^{32}$P labelled oligonucleotide probe, PR1 (Plate 5.2). 1fg represents approximately 62 copies of the viral genome. The sensitivity of PCR analysis was therefore approximately one thousandfold greater than that of dot-blot analysis.

5.1.3 Detection of HCMV RNA by PCR

5.1.3.1 Specificity

No specific amplification products were obtained when RNA PCR was performed on RNA extracted from cultured cells infected with the other human herpesviruses (HSV I, -II, EB, VZV, HHV-6) or total RNA extracted from uninfected HEL cells.

Two significant findings were made following RNA PCR of HCMV RNA extracted from both infected HEL cells and donor PBMC. Visualisation
Plate 5.2 Sensitivity of HCMV DNA PCR analysis. PCR analysis of known quantities - 500, 100, 50, 10, 5, 1, 0.5, 0.1 fg of pCM5018 DNA (lanes 2-9). A - reaction products electrophoresed on a 2% agarose Et/Br gel (lane 1 DNA size markers), B - S blot of A probed with $^{32}$P labelled CMPR1. Numbers on the LH side represent DNA size markers (bp).
Plate 5.3  PCR analysis of HCMV IE RNA. Lanes 1–3 RNA from AD169 infected cultured cells, lanes 4–5 PBMC RNA from HCMV-IgG positive donors, lane 6– RNA from uninfected HEL cells, lane 7– 2pg RNA from AD169 infected, cycloheximide treated, cultured cells, lane 8– DNA size markers. A – reaction products electrophoresed on a 2% agarose/EtBr gel showing: 227bp expected specific fragment (lanes 1–5, 7); approximately 200bp HCMV specific fragment (most visible in lanes 2 and 7); PCR-B (approximately 400bp) (lanes 1, 3–5). B – S.blot of A probed with $^{32}$P labelled CMPR2. The two specific hybridising fragments are demonstrated, whilst PCR-B does not hybridise with the probe. Numbers on the RH side represent DNA size markers (bp).
of RNA PCR amplification products on 2% agarose/EtBr gels showed the presence of two amplification products: the expected 227bp fragment together with a smaller fragment approximately 200bp in size. Visualisation of these two distinct fragments depended upon the electrophoresis conditions allowing sufficient resolution of the two fragments. The 200bp fragment usually appeared as a faint band on gels but hybridised with an intensity proportional to the 227bp fragment after Southern blotting of the gel (Plate 5.3). All samples analysed by RNA PCR that contained target RNA demonstrated this fragment in addition to the specific fragment, although visualisation on gels was not always apparent. Hybridisation with $^{32}$P labelled probe CMPR2 demonstrated the presence of the fragment in these instances.

A more significant finding was the appearance of an extra amplification product, termed PCR-B, following RNA PCR using primers CMP3/CMP4. The product was larger than the expected amplification product, approximately 400bp, compared to the expected 227bp, and it did not hybridise with the oligonucleotide probe, CMPR2, specific for the expected product (Plate 5.3). The formation of PCR-B appeared to have no adverse effect on the production of the specific fragment. Subsequently, it became apparent that in some way a low concentration of target nucleic acid seemed to favour the production of PCR-B concurrently with specific product. Thus PCR-B was only produced when target nucleic acid was present, and when that target was present in the RT reaction at an amount less than approximately 1pg, and was only visible when the reaction products were run on 2% agarose/EtBr gels (Plate 5.4).
Plate 5.4 Visualisation of PCR-B on an agarose gel. PCR was performed on known quantities - 50, 20, 10, 5, 1pg, 500, 100, 50, 20, 10fg of IE RNA extracted from AD169 infected, cycloheximide treated, cultured cells (lanes 2-11). Lane 1- DNA size markers. A - products of RNA PCR electrophoresed on a 2% agarose/EtBr gel. B - S.blot of A probed with $^{32}$P labelled CMPR2. An inverse relationship between the presence of PCR-B and the initial target concentration is clearly shown, but, as previously seen, PCR-B did not hybridise to the probe. Numbers on the LH side represent DNA size markers (bp).
Plate 5.5  Primer dependency of the production of PCR-B. PCR products from RNA PCR performed using single primers and the resulting products electrophoresed on a 2% agarose/EtBr gel. Lanes 2-4 and 5-7 represent the results of PCR analysis of PBMC RNA from 2 donors; lane 1- DNA size markers, lane 8- 2pg AD169 RNA. Lanes 2,5- PCR performed using only primer CMP4; lanes 3,6- PCR performed using only primer CMP3; lanes 4,7- standard PCR performed using both primers CMP3/CMP4. Numbers on the LH side represent DNA size markers (bp).
When RNA PCR was performed in the presence of target material, using the primer CMP4 in the standard RT reaction and not adding CMP3 prior to the PCR, the specific amplification product was not seen, but production of PCR-B still occurred (Plate 5.5).

The sequence of the specific 227bp RNA PCR amplification product was determined by Sanger dideoxy sequencing and the sequence of the DNA internal to the primers CMP3/CMP4 was found to agree with published sequence data (Sternberg et al, 1984). Attempts to clone and sequence the PCR-B fragment however, were unsuccessful. The fragment was similarly purified by agarose gel electrophoresis followed by electroelution, ligated into pUC18 which was then transfected into competent TG-2 cells. Although the fragment was successfully ligated into pUC18, more than 80 colonies were screened for the presence of clones containing the ligated plasmid, but none were found. Further attempts to clone and sequence the fragment were discontinued although it is hoped that the work will be continued at a later date.

5.1.3.2 Sensitivity

Using the antisense primer CMP4 for first strand priming, and then adding the sense primer CMP3 for the cDNA amplification, 0.1 fg of total RNA from HCMV AD169 infected, cycloheximide treated, HEL cells was detected on a 2% agarose/EtBr gel. This was confirmed by Southern blotting and probing with a $^{32}$P labelled oligonucleotide probe, CMPR2 (Plate 5.6). On the assumption that approximately 85% of the total RNA prepared from the cycloheximide treated infected cells is HCMV IE1 RNA, 0.1 fg of total RNA represents approximately 82 copies of HCMV IE1 RNA.
Plate 5.6 Sensitivity of RNA PCR. S blot of the reaction products of RNA PCR performed on known quantities - 1000, 500, 100, 50, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0 fg (lanes 1-12) of AD169 IE RNA, electrophoresed on a 2% agarose/EtBr gel and probed with $^{32}$P labelled CMPR2. Numbers on the LH side represent DNA size markers (bp).
Plate 5.7 Visualisation of unlabelled RNA transcripts from pCM01. Transcripts were electrophoresed on a 1.5% agarose/formaldehyde gel and stained with 0.5µg/ml EtBr. Lane 1- RNA size markers, lane 2- sense transcripts, lane 3- antisense transcripts, lanes 4-5 - transcription controls, sense and antisense transcripts of the human Ig gene RNA, lane 6- negative control, pCM01 sense transcription reaction without T7 polymerase. Human IgG RNA was transcribed from pA153 (sequence of human Ig gene DNA cloned into Bluescript+, provided by Dr N Short, Kings College, London). Figures on the LH side represent RNA size markers (Kbp).
5.1.4.1 Specificity of in vitro RNA Transcripts

Both sense and antisense RNA transcripts were successfully run-off from plasmid pCM01 and biotin labelled using CAB-NHS ester (Gibco-BRL). Analysis by gel electrophoresis demonstrated that the transcripts produced were approximately full length (Plate 5.7) but they were unfortunately found to be unsuitable as positive control material for use in RNA PCR, as the cloned fragment of HCMV AD169 DNA used to produce the transcripts was subsequently found not to contain the specific IE1 RNA sequence that is the target for the primer pair PIE1/PIE2. However, when labelled with biotin, the transcripts were considered suitable for use as highly specific hybridisation probes for the in situ detection of HCMV DNA and RNA in PBMC in future studies. The labelled transcripts did not give any signal when hybridised to DNA from uninfected HEL cells.

5.1.4.2 Sensitivity of in vitro RNA Transcripts

Biotin labelled sense and antisense transcripts were both found to detect 1pg of plasmid pCM5018 and 10pg of HCMV DNA dot-blotted onto nitrocellulose using the Blu-gene detection system (Gibco-BRL Life Sciences) according to the manufacturers instructions (Plate 5.8). These figures demonstrate the same sensitivity as that obtained using the $^{32}$P labelled DNA probe. Labelled antisense transcripts were able to detect approximately 10fg of total cellular RNA from HCMV AD169 infected, cycloheximide treated, HEL cells dot-blotted onto nitrocellulose (Plate 5.9). On the basis of calculations made in section 5.1.3.2 above, detection of this quantity of HCMV RNA represents the detection of approximately 8200 copies of HCMV IE1 RNA.
Plate 5.8  Sensitivity of DNA dot-blot analysis using biotin-labelled RNA transcripts as hybridisation probes. Known amounts of pCM5018 lanes 1-2, 4-5, and HCMV DNA lanes 3,6 were dot-blotted onto nitrocellulose and probed with biotin-labelled, pCM01 sense (lanes 1-3) and antisense (lanes 4-6) transcripts. A- 50pg, B- 10pg, C- 5pg, D- 1pg, E- 0.5pg, F- 0pg of DNA. Visualisation of the hybrids was achieved using the Blu Gene DNA detection system (Gibco-BRL).
Plate 5.9 Sensitivity of RNA dot-blot analysis using biotin-labelled RNA transcript as the hybridisation probe. Known amounts of AD169 IE RNA - 1pg, 500, 100, 10, 1, 0fg (A-F) were blotted onto nitrocellulose and probed with biotin labelled pCM01 antisense transcript. Visualisation of the hybrids was achieved using the Blu Gene DNA detection system (Gibco-BRL).
Dot-blot analysis was performed using the *Eco* RI J fragment of AD169 (propagated in plasmid pCM5018) as the hybridisation probe. Although initially the *Hind* III L fragment of AD169, cloned as pBW3, was obtained and prepared for use as the hybridisation probe, this was replaced with the *Eco* RI J fragment, which originates from a region of the genome coding for the major IE RNA of HCMV and was therefore thought to increase the probability of detecting any specific NA, enabling detection of both HCMV RNA and DNA.

Although biotin labelling of the fragment was attempted using both biotin-11-dUTP (Gibco-BRL) and Photo-Biotin (Vector Labs), the results were not encouraging, due to low specific activity and corresponding low sensitivity of the probe. Consequently dot-blot analysis was only performed using the $^{32}$P labelled fragment.

The specificity of the probe was found to be high and non-specific hybridisation with human genomic DNA did not occur. Previous studies have shown that the region of the HCMV genome from which the fragment is derived does not show homology to human genomic DNA (Ruger *et al*, 1984). The non-specific background hybridisation signal that often occurs when screening blotted samples containing fairly high concentrations of nucleic acid was reduced considerably by prehybridising the filters for 24 hours prior to hybridisation and by using extended washing procedures. These measures did not significantly affect the strength of any hybridisation signals, the technique routinely detecting 0.5-2pg, generally 1pg, of plasmid pCM5018 in reconstruction experiments.
Although the use of hybridisation techniques enables the detection of specific target NA and refinements have increased the sensitivity so that picogram/femtogram amounts of NA can now be detected routinely, when considered in terms of number of genome copies detected, it is clear that even greater sensitivity is desirable. The dot-blot procedure and hybridisation techniques used in this study enabled the detection of 0.5-2 pg of plasmid pCM5018 DNA, equivalent to approximately 3.1 x 10^4 to 1.24 x 10^5 copies of the HCMV gene, and detection of 10 pg of AD169 HCMV DNA equivalent to approximately 3.8 x 10^4 copies of the HCMV genome. These two values are (and should be) similar, and further validate the performance of the analysis. The use of both prepared test strips bearing known amounts of HCMV DNA, and the incorporation of dilutions of pCM5018 DNA onto most of the dot-blot filters prepared, provided standardisation and quality control both in terms of the specific activity of the probe, and the hybridisation and washing conditions. Slight variations were seen on some filters. Indeed, one set of filters gave virtually no hybridisation signal from the diluted pCM5018 DNA although the positive control and the separate test strip gave the expected signals. The pCM5018 stock was subsequently found to have been contaminated, presumably with a nuclease, and the DNA concentration had decreased.

As stated above, greater sensitivity may be desirable. The amount of HCMV excreted in urine, for example, can vary greatly, with titres as low as 10-100 pfu/ml being recorded. On this basis, and on the (unlikely) assumption that all viral particles present were infectious in in vitro culture, in vitro detection of 10 ag of DNA would be necessary to detect viral DNA in such a sample. Dot-blot sensitivities currently do not reach such a high level.
When reconstruction experiments were performed using pCM5018 in the presence of added HEL cell DNA, no significant difference in the sensitivity of the technique was observed. Interestingly, Saltzman et al. (1990) reported a 10-fold reduction in the sensitivity of hybridisation of the Xba I C fragment of the Towne strain of HCMV to purified Towne HCMV DNA (from 1pg to 10pg) when performed in the presence of 10μg of PBMC DNA. The sensitivity of detection of AD169 HCMV DNA reported here is 10pg, a figure that agrees with that of Saltzman as an absolute level of detection of HCMV DNA by dot-blot analysis. Furthermore, the AD169 DNA used in this study was prepared from infected cells and was therefore contaminated with a small amount of host cell DNA.

However, the use of dot-blot analysis does provide a convenient and rapid method (compared to conventional cell culture) for the examination of potentially large numbers of samples for the presence of target NA. Both infectious and non-infectious NA can be detected and the level of sensitivity defined and maintained.

It is clear that the use of PCR, if performed correctly, not only provides a significant increase in sensitivity but also a technique that can provide reliable results in a fraction of the time taken by more conventional hybridisation techniques. With a working sensitivity of 1fg of pCM5018, representing approximately 62 genome copies, the DNA PCR technique, although not quantitative, was $10^3$ times more sensitive than conventional dot-blot analysis.

A major problem associated with PCR is contamination. Sources of contamination include the products of previous reactions, since large amounts of specific fragment are produced. In this study, strict
regimes were implemented to prevent contamination but these failed on a number of occasions and contamination became a major problem for a period of time. Subsequently problems were overcome by the controlled production of large batches of reagents, the preparation of premixed reaction mixtures, the single use of aliquoted reagents and restriction endonuclease digestion of primers prior to use. In addition, dedicated pipettes, disposables and work areas were maintained. All subsequent work was then carried out without any further contamination problems.

Standardisation of the PCR technique has been shown to be necessary to ensure optimal efficiency (Saiki, 1989), and is an important requirement when detection of low levels of target is required. The use of unpurified oligonucleotide primers is an area of discussion, with some authors advocating purification by HPLC or PAGE to remove all short length oligonucleotides and others finding that the quality of oligonucleotide primers synthesised on modern synthesisers was more than adequate. All primers used in this study were used unpurified without experiencing any untoward problems. They were, however, restriction enzyme digested as a means of reducing contamination of the primers with specific DNA. The fragment amplified by the DNA primers P1/P2 contains a single Taq I restriction site, predigestion of the primers with Taq I thus eliminating any contaminating target sequences. No adverse affects of this action were seen on the sensitivity of the PCR analysis.

The Mg^{2+} concentration in the reaction buffer was optimised for each pair of primers. Despite the measures taken to optimise the technique, there was often noticeable variation between experiments, even when the same samples were used. Variation in the intensity of the
amplification products and background fluorescence on visualisation after electrophoresis through 2% agarose/EtBr was particularly evident. A further finding was the appearance of 'ladders' of non-specific bands, both larger and smaller than the specific product, but associated only with reactions containing target NA. The single factor responsible for this variation was found to be the Taq polymerase enzyme. Only the Taq polymerase, Amplitaq (ILS, Perkin-Elmer Cetus) was used in the reactions, and although this is a cloned enzyme, batch to batch variation was clear affecting the specificity more than the sensitivity. Non-specific bands were clearly identified by their inability to hybridise to the oligonucleotide probes internal to the specific fragments.

The amplification products from RNA PCR generally appeared as more intense and well-defined bands than DNA PCR when visualised on 2% agarose/EtBr gels. The formation of specific amplification products consisting of the two hybridising fragments was seen with all samples containing amplifiable target RNA. The reasons for the formation of the smaller fragment are unclear, although the hybridisation of probe CMPR2 to the fragment demonstrates its specificity. The origins of the fragment may lie in the degeneration or splicing of specific full-sized fragments or conceivably in the amplification of a separate RNA transcript, either a degenerate spliced form of the target or a different transcript, but with sufficient sequence homology to allow complementarity of the primers and thus cDNA synthesis and amplification. It is unlikely that the second explanation is viable; a degenerate, spliced form of the target RNA with a length of NA excised or excised and replaced with a shorter sequence but internal to the
primer sites is a more probable explanation. Only sequence analysis will be able to demonstrate clearly the origin of the fragment.

The amplification of the additional PCR product (PCR-B) following RNA PCR was an entirely unexpected finding and initially of great interest. Initially it was considered possible that, if the product was a genuine amplification product and was specific to HCMV, it might represent a transcript associated with the induction and/or maintenance of latency. The failure of the oligonucleotide probe CMPR2 to hybridise to PCR-B, although not conclusive, seemed to indicate that the fragment was probably not specific to HCMV and was more likely to be a non-specific product. It is not likely that PCR-B contains intron sequences as the primer pair CMP3/CMP4 was selected from within a single exon (exon 4, Sternberg et al, 1984). The probe CMPR2 would, therefore, only hybridise to the exon sequence. The consistent finding of PCR-B produced by RNA PCR amplification of PBMC RNA, but not always on amplification of RNA produced from cell culture, was puzzling. An explanation for this was found when the sensitivity of the RNA PCR technique was being studied using diluted HCMV AD169 IE RNA which showed an apparent inverse relationship between the initial target RNA concentration and the appearance of PCR-B and an approximate cut-off point, in terms of initial HCMV RNA concentration, above which PCR-B was not produced. Conceivably, above the required level the amount of target present utilises the primers and dNTP's to the exclusion of the formation of PCR-B. Below this concentration, production of PCR-B is facilitated.

Interestingly, PCR-B was still produced following RNA PCR using only the antisense primer where the specific product was not produced. When this experiment was repeated using only the sense primer for
RNA PCR, no products were apparent. These findings would seem to indicate that PCR-B is most probably an artefact produced as a result of interaction during the RT or PCR or both parts of the RNA PCR procedure, but only involving the antisense primer. When the data are analysed it appears that the production of PCR-B is affected by the following events: interaction of the antisense RNA PCR primer (CMP3) and HCMV IE RNA occurring at some point during the procedure; an absolute requirement for target RNA, but the presence of target at a level higher than approximately 1pg in some way inhibits production of PCR-B.

In a final attempt to resolve the situation, DNA sequencing of both the specific product and PCR-B was attempted. The sequence of the specific fragment internal to primers CMP3/CMP4 was successfully determined and matched published sequence data. Attempts to clone PCR-B were, however, unsuccessful. It is envisaged that further attempts to clone and sequence the fragment will be made in the near future as only this will provide the definitive evidence needed to explain the origin of and events leading to the production of PCR-B. It is, therefore, highly probable that the fragment is an artefact, production of which requires priming on HCMV RNA, but which may consist of either primer sequencies only or of non-specific sequences initiated by the primer.

RNA transcripts were produced from clone pCM01 in large quantities, were conveniently labelled with biotin to a high specific activity and gave good results when hybridised to dot-blotted RNA and DNA. The unlabelled transcripts were also suitable substrates for RNA PCR using DNA primers CMP1/CMP2. It is, therefore, rather unfortunate that the incorrect region of the HCMV genome was selected for cloning into
Bluescript, and that the RNA transcripts subsequently produced were unsuitable as control RNA for RNA PCR using the IE1 specific primers CMP3/CMP4. Selection of the sequence for cloning was made on the basis that the DNA PCR primer pair (CMP1/CMP2) was complementary to a sequence of HCMV DNA which included the region containing exon 2 which transcribes to IE1 RNA, and the primers would thus serve as suitable primers for RNA PCR as well as DNA PCR. This was subsequently shown not to be the case, the primer pair was actually complementary to a sequence found in exon 5 which is transcribed to IE2 RNA. Problems were encountered during development of the RNA PCR technique when in vitro RNA transcripts from pCM01 gave expected specific amplification products, whilst cycloheximide induced HCMV IE1 RNA failed to amplify. RNA primers CMP3/CMP4 were subsequently designed using published sequence data (Sternberg et al, 1984) in order to amplify a suitable region of HCMV IE1 exon 4.

Biotin labelling of the RNA transcripts was achieved using a two-stage procedure, firstly by incorporation of allylamine-UTP into the transcripts in the place of rUTP and, secondly, biotinylation by chemical modification using CAB-NHS ester. The direct incorporation of biotinylated nucleotide into the transcripts was not possible due to the temporary unavailability of commercial biotin-11-rUTP. Although the use of allylamine-UTP required an extra step, the chemical coupling reaction was simple and straightforward to perform and, more importantly, the use of allylamine-UTP results in a greater yield of RNA than when rUTP is used (Gibco-BRL Allylamine-UTP product data sheet).

The results of dot-blot hybridisation using the biotin labelled transcripts as hybridisation probes do, however, demonstrate the
potential of plasmid pCM01 to produce RNA probes suitable for in situ hybridisation studies. These will be used to investigate the presence of HCMV DNA in PBMC from HCMV-IgG positive and negative blood donors in future studies.
CHAPTER 6  HCMV VIRAEMIA - SERUM ANALYSIS

6.1 RESULTS

6.1.1 Dot-blot Analysis of Donor Serum Samples

Serum samples from a total of 1445 HCMV-IgG positive donors and 833 HCMV-IgG negative donors were screened for the presence of HCMV DNA by dot-blot analysis. None of the samples were positive.

6.1.2 Dot-blot Analysis of Clinical Serum Samples

Dot-blot analysis was performed on serum samples obtained from a total of 35 patients with active or recent HCMV infection (Table 6.1). Five of these samples demonstrated clear hybridisation and were therefore considered to contain HCMV DNA (Plate 6.1).

Table 6.1 Clinical Details of Patients With Serum HCMV DNA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis/Details</th>
<th>Serology</th>
<th>Infection type¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>Pregnant, Pyrexial illness (2/52)</td>
<td>Pos Pos</td>
<td>Primary</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>Renal transplant (5/52) [HCMV positive kidney]</td>
<td>Pos Pos</td>
<td>Primary</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Glandular fever like illness (3/52)</td>
<td>Pos Pos</td>
<td>Primary</td>
</tr>
<tr>
<td>4</td>
<td>4 months</td>
<td>Congenital</td>
<td>Pos Pos</td>
<td>Primary</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>Renal dialysis patient</td>
<td>Pos Pos</td>
<td>Reactivation/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reinfecion</td>
</tr>
</tbody>
</table>

¹ infection type based upon clinical and laboratory findings and supplied by the laboratory providing the samples
Plate 6.1 Dot-blot analysis of serum DNA from acute phase clinical samples. Lanes 1-6, A-C, dot-blotted serum samples from 6 patients with acute HCMV infection. Prepared samples were blotted in volumes of A- 400μl, B- 200μl, C- 100μl. 1D- 20pg HCMV DNA, 2D- 1μg uninfected genomic DNA, 3D-6D known quantities - 5, 2, 1, 0.5pg, of pCM5018. The blots were probed with $^{32}$P labelled Eco RI J fragment of HCMV.
6.1.3 DNA PCR Analysis of Donor Serum Samples

Serum samples from a total of 150 HCMV-IgG positive donors and 100 HCMV-IgG negative donors, previously screened for HCMV DNA by dot-blot analysis, were further screened for the presence of HCMV DNA using PCR analysis. None of the samples demonstrated specific amplification products.

6.1.4 DNA PCR Analysis of Clinical Serum Samples

PCR analysis was performed on the DNA extracted from 23 of the clinical samples. Three of these samples were amplified yielding a product of the expected size (Plate 6.2). These results were in complete agreement with the dot-blot analysis results previously obtained from these samples (Section 6.1.2). Only 23 of the 35 clinical samples were tested as there was insufficient sample available. The samples tested only included 3 of the 5 samples found to be dot-blot positive.
Plate 6.2  PCR analysis of serum DNA from acute phase clinical samples. The reaction products were electrophoresed on a 2% agarose/EtBr gel. Lanes 1-6 samples 1-6, lane 7- uninfected genomic DNA, lane 8- 5pg HCMV DNA. Numbers on the LH side represent DNA size markers (bp).
6.2 DISCUSSION

The detection of free HCMV in the sera of a number of patients with acute HCMV infection is important and may have implications for transfusion practice. That none of the donor serum samples were found to contain detectable HCMV DNA accords with what would be expected for healthy individuals, even if latently infected, but does not rule out the possibility of an occasional donation from a viraemic donor.

Samples obtained from a number of patients with proven acute or recent HCMV infection have been used throughout this study as controls or to validate techniques, although the results obtained with these samples have not been reported as they are not representative of infections in immunocompetent individuals. It was felt, however, that these particular results were of significance as they demonstrated the existence of a potential stage of infection that might be found in immunocompetent individuals and so should be considered. The transfusion of blood from such a donor could result in the transmission of HCMV; furthermore the transfusion of plasma products from such a donor could also result in the transmission of infection though this is a route not normally considered to be associated with any risk of transmission of HCMV. That any transmission by this particular route is rare is demonstrated by the findings of Adler (1988) and Bowden & Sayers (1990), both of whom studied the transmission of HCMV infection by FFP and concluded that it did not transmit HCMV infection.

HCMV viraemia commonly occurs in immunosuppressed individuals and may indicate a systemic disease which is often associated with a poor
prognosis. Detection of viraemia, however, is achieved by the isolation or identification of virus or viral DNA solely in the leucocytes of the infected individual. Detection of virus free in serum has not been reported in any published studies. There can be little doubt, however, that acute infection in any individual could include a period of viraemia, with both cell-associated and free virus being present in the circulation; such a condition is clearly much more common in immunocompromised individuals than in blood donors. Interestingly, one of the five dot-blot positive clinical samples (donor 3) was an immunocompetent individual suffering a mild glandular fever-like illness; virus serology and clinical picture being consistent with primary infection.

An important, but largely unresolved, question is whether the free virus represents virus release into the blood from infected leucocytes or is the source of virus that infects the leucocytes or, indeed, whether both situations occur. Although cell to cell contact is the usual mechanism of spread of HCMV infection, infection of discrete cells such as leucocytes must either occur at an earlier phase of cell development or involve infection of the mature cell by free virus. The length of time that viraemia can be detected in acutely infected individuals suggests that infection of mature cells occurs. In vitro infection of leucocytes with free HCMV has been demonstrated although only restricted infection was seen (Einhorn & Öst, 1984).

That the release of detectable levels of free virus does not occur very often or occurs for only a very short period is shown by the lack of detection of free virus in the sera of HCMV-Ab positive and negative donors in this study together with the failure to identify transmission of infection by FFP (Adler, 1988; Bowden & Sayers, 1990).
CHAPTER 7  HCMV VIRAEMIA - PERIPHERAL BLOOD MONONUCLEAR CELLS

7.1 RESULTS

7.1.1 Dot-blot Analysis of PBMC NA

PBMC NA from 1462 donors was analysed by dot-blot analysis. 982 of these donors were HCMV-IgG positive (seropositive) and 480 were HCMV-IgG negative (seronegative). HCMV DNA could not be detected in any by hybridisation.

7.1.2 PCR Analysis of PBMC DNA

PCR analysis of PBMC DNA was performed using primer pair CMP1/CMP2 (Plate 7.1). Satisfactory analysis was performed on PBMC DNA samples from a total of 205 blood donors, of whom 102 were seropositive and 103 were seronegative. Specific products (240bp) were demonstrated following amplification of the DNA from 95 of the donor samples (PCR positive), of which 84 were from seropositive and 11 from seronegative donors. No products were found after amplification of the DNA from any of 110 donor samples (PCR negative), of which 92 were from seronegative and 18 from seropositive donors (Table 7.1). 18 of the 102 seropositive donors (17.6%) were PCR negative whilst 11 of the 103 seronegative donors (10.8%) were PCR positive.
Plate 7.1  PCR analysis of DNA extracted from donor PBMC samples. Reaction products were electrophoresed on a 2% agarose/EtBr gel, blotted and probed with $^{32}$P labelled CMPRI. Lanes 1-10 PBMC DNA samples, lane 11- uninfected genomic DNA, lane 12- 1pg HCMV DNA. Numbers on the LH side represent DNA size markers (bp). The HCMV-IgG status is indicated along the bottom.
Table 7.1 Correlation Between HCMV Serostatus and DNA PCR analysis

<table>
<thead>
<tr>
<th>HCMV Ab STATUS</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR STATUS</td>
<td>84</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>92</td>
</tr>
</tbody>
</table>

n=205

When visualised on 2% agarose/EtBr gels the amplification products from some PBMC samples appeared as distinct but low intensity bands, although they were clearly demonstrated following Southern blotting and hybridisation using the $^{32}$P labelled oligonucleotide probe CMPR1. Conversely, some samples gave very clear strong bands when visualised on the stained gels.

On some occasions, satellite DNA bands of a smaller size than the specific product were seen. None of these bands hybridised with CMPR1. The presence of satellite bands did not appear to have any detrimental effect on production of the specific product. The presence of high background fluorescence on the gels with some of the reactions was generally associated with the use of PBMC DNA prepared by direct lysis of the cells in PCR buffer, rather than by conventional extraction procedures.

7.1.3 Nested DNA PCR Analysis

The nested primer pair CMN1/CMP2 resulted in a 194bp fragment when used for a second round of PCR on the amplification products of DNA PCR using primer pair CMP1/CMP2 (Plate 7.2). Southern blotting and hybridisation of the products of nested PCR was possible as the
Plate 7.2  Nested PCR analysis. PBMC DNA samples were subjected to standard PCR (A) and then nested PCR analysis (B). Both sets of reaction products were combined and electrophoresed on a 2% agarose/EtBr gel, blotted and probed with $^{32}$P labelled CMPR1. Lanes 1-8 PBMC DNA samples, lane 9- uninfected genomic DNA, lane 10- 1pg HCMV DNA. The weak result seen in lane A3 from the initial PCR is clearly seen following nested PCR (B3). Numbers on the LH side represent DNA size markers (bp). The HCMV-IgG status is indicated along the bottom.
amplified fragment also contained the sequence complementary to probe CMPR1 and this did not overlap the internal primers.

This further analysis was used to demonstrate the specificity of the initial PCR. A total of 36 PCR reactions (19 PCR positive and 17 PCR negative) were analysed by this method. None of the initially negative PCR reactions were subsequently determined to be PCR positive. The 17 PCR negative samples analysed included 16 of the 18 HCMV-IgG positive, PCR negative donors. All of the initially PCR positive reactions were confirmed as positive using nested PCR.

7.1.4 DNA PCR Analysis of Human β-Globin Gene

PCR analysis of PBMC DNA using the β-globin primer pair BEP1/BEP2 was performed under the same conditions as the amplification of PBMC HCMV DNA, except that only one tenth of the amount of genomic DNA was used in the reaction. Amplification of all 18 of the HCMV-IgG positive/PCR negative samples gave specific 110bp product demonstrating that there was sufficient PBMC DNA present in the volume of sample analysed, and that the DNA was a suitable substrate for PCR and for detection of single copy sequences (Plate 7.3).

7.1.5 Confirmation of the HCMV-Antibody Status of PCR Positive/HCMV-Antibody Negative Donors

The IB results from HCMV PCR positive but HCMV-Ab negative donors confirmed the Ab status of the donors. Some samples produced weak bands but no donors demonstrated the 150/55 kDa bands required to confirm HCMV specificity. Repeat HCMV-Ab screening of the samples using the same or a different commercial EIA, together with the
Plate 7.3  β-globin PCR analysis of PBMC DNA from the HCMV-IgG positive/DNA PCR negative donors. Reaction products were electrophoresed on a 2% agarose/EtBr gel. Lanes 2-13 and 17-22 PBMC DNA, lanes 14,23- non-human DNA, lanes 15,24- genomic DNA, lanes 1,16- DNA size markers (numbers on the LH side [bp]).
Table 7.2 Correlation of HCMV serostatus, DNA PCR and RNA PCR analysis

<table>
<thead>
<tr>
<th>A HCMV-IgG STATUS</th>
<th>B DNA PCR</th>
<th>C RNA PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>20 4</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>20 21</td>
</tr>
<tr>
<td>RNA PCR +</td>
<td>RNA PCR +</td>
<td>24 0</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>12 29</td>
</tr>
</tbody>
</table>

A Correlation of HCMV serostatus and RNA PCR analysis
B Correlation of DNA PCR and RNA PCR analyses

'in house' HCMV-IgM and -IgA assays, also failed to demonstrate any HCMV-Ab in the sera from these donors.

7.1.6 PCR Analysis of PBMC RNA

RNA PCR was performed on total RNA extracted from PBMC from 65 donors, 40 of whom were seropositive and 25 seronegative. The results of RNA and DNA PCR analysis on the same donor samples were examined to correlate serostatus, DNA PCR result and RNA PCR result (Table 7.2). Specific product (227bp) was obtained following amplification of PBMC RNA from 24 of the 65 donors. Four of the 25 seronegative donors demonstrated specific product following amplification, whilst 20 of the 40 seropositive donors gave no amplification product. Comparison of the DNA and RNA PCR results demonstrates correlation between the results; all 24 of the RNA PCR positive donors were also DNA PCR positive, although only 24 of the 36 DNA PCR positive donors were RNA PCR positive. Confirmation of the specificity of the product was achieved by Southern blotting and hybridisation with $^{32}$P labelled oligonucleotide probe CMPR2 (Plate 7.4). The unexpected amplification of the RNA PCR product PCR.B has been discussed previously in section 5.1.3.1.
Plate 7.4  PCR analysis of PBMC RNA. S blot of the reaction products of RNA PCR electrophoresed on a 2% agarose/EtBr gel and probed with $^{32}$P labelled CMPR2. Lanes 1-12 PBMC RNA, lane 13– uninfected cell RNA, lane 14– 2pg HCMV IE RNA. Numbers on the LH side represent DNA size markers (bp). The HCMV-IgG status is indicated along the bottom.
7.2 DISCUSSION

Involvement of leucocytes in acute HCMV infection has been recognised for some time; viraemia marking dissemination of virus. A number of important aspects concerning infection of leucocytes are still unclear and include the type of cell infected, the initial site of infection, the extent of replication of the virus in these cells and the mechanisms by which latency may subsequently be established. Whilst the role of the leucocyte as a site of HCMV latency has largely been inferred from observations of transmission of infection via transfusion, until recently there was little molecular evidence to support these hypotheses. The ability to culture HCMV from the leucocytes of an infected individual does not in itself implicate these as sites of latency, since virus may be present due to the phagocytic activity of some of the cells. There is only a single report of the successful culture of HCMV from the leucocytes of HCMV-Ab positive donors (Diosi et al, 1969) and it is quite possible that these donors were at a late stage of acute asymptomatic infection when bled, where sufficient virus remained to be detected by culture.

Detection of viral NA, by dot-blot hybridisation, in PBMC from both HCMV-IgG positive and negative donors was totally unsuccessful and demonstration of latent virus was not achieved. Even though the hybridisation probe used was specifically chosen to include sequences capable of hybridising to both HCMV-DNA and -IE RNA, there was apparently insufficient target material for detection. The analysis was performed on NA from a total of approximately $10^5$ cells, the sensitivity of the analysis being determined as 0.5-2pg of plasmid pCM5018 (routinely 1-2pg). This quantity of DNA (1pg) is equivalent to approximately 62,000 copies of the genome. Analysis was performed on
the DNA extracted from 2 x 10^5 cells and, therefore, to obtain even the weakest specific dot-blot result, the presence of a single viral genome in at least 15-5% of the cells would be required. Such a large quantity of virus is unlikely to be associated with a latent infection. If the level of infection with latent virus is in the range of a single genomic copy in 0.01-1% of PBMC, as suggested by Schrier et al (1985) and Nelson et al (1990), dot-blot analysis would be required to detect as few as 10-1000 copies of genome, which is approximately 0.2-20 fg of PCM5018 DNA, a level of sensitivity which cannot be attained by this technique. Nonetheless, detection of viraemia is possible by this technique. Numerous studies have involved use of the method to demonstrate the presence of HCMV DNA and RNA in the leucocytes of acutely infected individuals (Saltzman et al, 1990; Dankner et al, 1990). The technique is, however, too insensitive to detect latent HCMV NA in PBMC from immunocompetent individuals.

The failure of dot-blot analysis to detect any HCMV NA in PBMC DNA demonstrated the need for a far more sensitive technique if detection of latent HCMV was to be successful. Subsequently, PCR has been used to analyse the PBMC DNA and in this study DNA from the PBMC of a total of 239 HCMV-IgG positive and -negative donors was subject to successful DNA PCR analysis. The ability of PCR to detect very low levels of target nucleic acid, often in the presence of large amounts of genomic or other foreign nucleic acid, is ideally suited for the detection of latent viruses.

The detection of HCMV DNA in PBMC from immunocompetent individuals provides molecular evidence to support the contention that virus is present, probably in a latent state, in PBMC from HCMV-Ab positive donors and that this is at least one source of transfusion transmitted
infection. Furthermore, the detection of DNA in PBMC from HCMV-Ab negative donors clearly demonstrates the presence of potentially infectious virus in donors considered to present no risk of transmitting HCMV. Additionally, a number of HCMV-Ab positive donors were found to be PCR negative. Although considered to be at risk on the basis of HCMV-Ab status, it is uncertain whether these were donors in whom latency had not established or whether virus was present at a level lower than normal, and thus undetectable by this PCR procedure.

The sensitivity of the technique, approximately $10^3$ times greater than dot-blot analysis, represents a major improvement and was sufficient to detect as few as 62 copies of the HCMV genome. As PCR analysis was performed on DNA prepared from approximately $5 \times 10^5$ PBMC, the technique was sufficiently sensitive to detect the presence of 1 copy per 8065 (0.012%) cells. This figure agrees with those reported by Schrier et al (1985) and Nelson et al (1990).

A second round of PCR using nested primers has been used by many authors to confirm the specificity of the products from the first analysis, especially in situations where only a small quantity of product was formed, or when a number of non-specific bands or high backgrounds were present. In this study true nested PCR was not performed but, rather, PCR products were further analysed using a nested upstream primer (CMN1) internal to CMP1, together with the original downstream primer (CMP2). No discrepancies were seen between the initial and nested results following further analysis of the initial product using the primer pair CMN1/CMP2. Although the use of further rounds of amplification gives increased sensitivity, the risk of contamination also increases such that some initial reaction mixes that
were contaminated with only minute amounts of target and though not amplified sufficiently to be detectable after initial amplification would be sufficiently amplified and detectable following the second round of PCR.

Preparation of sample DNA for analysis is greatly simplified if crude DNA preparations can be used. Cell lysates prepared by lysis and digestion of the proteins followed by heat inactivation of any DNases and pelleting of cellular debris are suitable DNA substrates for PCR. This provides a saving in the time and amount of work required and also cuts down the number of manipulations, so decreasing the risk of contamination. In this study, both conventional DNA extraction and rapid cell lysis techniques were used with no significant difference in amplification efficiency. Efficiency was found to be decreased, however, in certain lysate preparations which contained high levels of haemoglobin, generally resulting from red cell contamination of the PBMC layer following density-gradient separation. A similar finding was reported by Higuchi (1990) who further demonstrated that the porphyrin compounds derived from haemoglobin were the most inhibitory substances found in blood.

In view of these findings it was felt that there was a possibility that the lack of specific amplification product in a number of HCMV-Ab positive individuals could be due to inhibition of PCR rather than to a lack of target DNA. That this was not the case was clearly shown by the use of PCR to amplify β-globin gene DNA in the PBMC DNA preparations from the 18 PCR negative HCMV-Ab positive donors. Production of the specific 110bp product in all 18 PCR reactions
demonstrated both that there was sufficient DNA present and that the DNA was a suitable substrate for PCR analysis.

The finding of PCR amplification products in 84 of 102 HCMV-Ab positive and 11 of 103 HCMV-Ab negative donors supports those of other authors who have detected HCMV NA in the PBMC of immunocompetent individuals (Cassol et al, 1989; Schrier et al, 1985; Nelson et al, 1990). There is, therefore, a population of donors who would be considered HCMV-Ab negative according to conventional serology but in whom the presence of viral DNA can repeatedly be demonstrated. Possible interpretations of these results include incorrect interpretation of the serology. Whilst most commercial HCMV-Ab assays are reliable, a situation may exist whereby a weakly reactive sample was screened as HCMV-Ab negative. However, this was not the case in this study since no HCMV-Abs were detected upon repeat testing or following IB. A second possibility is that donors may be HCMV-Ab positive but with Ab levels which have dropped below those routinely detectable. Finally, there exists a small population of donors who do not mount an immune response to HCMV, although latency may be established following recovery from acute infection.

The major significance of this observation is the identification of a potential source of infection from apparently low risk donations, though quantification of this risk is virtually impossible without prospective study of HCMV infection rates in immunocompromised patients with thorough investigation of any cases of transmission of HCMV by units screened as Ab negative.

In order to determine whether the DNA detected was transcriptionally active, PCR analysis was performed on total RNA prepared from donor PBMC using a primer pair specific for HCMV IE1 RNA (CMP3/CMP4). IE
RNA could not be detected in all of the DNA positive donors and was detected in only 66% of these cases. IE RNA was not detected in the PBMC of any DNA PCR negative donor.

The mechanisms controlling transcription of RNA from latent HCMV DNA are poorly understood, and several mechanisms may combine to produce a variety of patterns in different individuals. The detection of IE RNA demonstrates that transcription of latent viral genomes is possible and it is therefore quite feasible that these are a source of infection transmitted by transfusion. Data obtained during this study supports that of Schrier et al (1985) who used in situ hybridisation to demonstrate the presence of HCMV IE RNA in PBMC from immunocompetent individuals. This technique also enabled the approximate number of cells expressing IE RNA to be determined, ranging from 0.035-2%. The RNA PCR technique used in this study was capable of detecting approximately 0.1 fg of RNA, i.e. 82 copies of IE1 RNA, which equates to the detection of at least one copy of IE RNA per 6098 (0.016%) cells, a value similar to that of Schrier et al (1985).

That HCMV-RNA was not found in the PBMC of all of the DNA PCR positive donors may be due to a failure of detection rather than to the absence of transcription. Possible explanations for the failure to detect IE RNA by PCR may include the loss of RNA during the preparation procedure and failure to produce cDNA suitable for PCR in the RT reaction. Conventionally, the isolation and purification of RNA is a time consuming procedure with great care being needed to prevent degradation by endogenous RNases. The method used in this study (Chomczynski & Sacchi, 1987) is a far more practical procedure which relies upon the presence of guanidinium thiocyanate during most of the procedure to denature any RNases. Whilst not resulting in the
purity obtained from density gradient preparations, the method has the ease of handling needed for large sample numbers and is suitable for use with small amounts of tissue. The quality of the RNA prepared was shown to be suitable for reverse transcription and therefore for the RNA PCR technique. It is quite probable that, in some of the RNA preparations, the presence of a contaminating agent may have inhibited either the RT reaction or the PCR itself, resulting in failure to amplify the target sequence.

The absence of detectable RNA may be due either to the absence of transcription, with either a complete absence or an intermittent transcription pattern, or to continued transcription of RNA at levels below those detectable by PCR. In a number of individuals who possess latent virus, it is possible that no transcription of the viral DNA occurs. In such cases, host immune mechanisms may be involved in regulation of transcription. A situation of intermittent, restricted transcription is possible during latent infection if the immune mechanisms which prevent recrudescence of the agent are not constantly effective but instances of the mechanisms failing or being overcome occur, leading to periods when limited transcription of the DNA are possible.

The possibility that insufficient RNA is produced for detection is unlikely. Detection of latent DNA has already been demonstrated (Stanier et al, 1989; Nelson et al, 1990). Transcription of RNA from the DNA would invariably result in multiple copies of the RNA being produced from each viral genome. Theoretically this would result in a significantly larger amount of target being present than that for DNA
PCR. However, RNA PCR may be less sensitive than DNA PCR as the RT reaction is usually only 5-50% efficient.

Although the presence of HCMV NA in PBMC from immunocompetent individuals has been detected by PCR, this technique does not permit identification of the specific cell type(s) harbouring the latent virus. Only if absolutely pure preparations of each class of mononuclear white blood cells can be achieved, can PCR be used for this. If the data obtained in this study are combined with the *in situ* hybridisation data from Schrier *et al* (1985) and Nelson *et al* (1990) sufficient information concerning latency exists to state that: a small proportion of PBMC from immunocompetent HCMV-Ab positive and negative individuals contain latent HCMV DNA, limited expression of IE RNA occurs in these cells and the types of cell infected are monocytes and, to a lesser degree, T-lymphocytes.

Studies performed on leucocytes from acutely infected and viraemic patients showed that the infected cell types were different from those in which the virus may become latent. From studies applying MoAbs to HCMV proteins (Revello *et al*, 1989; Gerna *et al*, 1990) or *in situ* hybridisation to HCMV RNA and DNA (Dankner *et al*, 1990), the predominant cell type infected during acute infection was shown to be the polymorphonuclear leucocyte (PMNL); infected monocytes were also seen but lymphocytes were rarely infected. The presence of HCMV in PMNL is generally considered to be due either to persistent infection of precursor cells in the bone marrow or to result from phagocytosis of entire virions by circulating cells. The demonstration of HCMV IE, E and L RNA transcripts indicates that there is active expression and perhaps replication of virus. The initial site of infection of PMNLs is currently unknown. Phagocytosis of entire virus does not alone
explain the production of RNA, and presumably a productive infection must ensue. Indeed, *in vitro* studies have produced limited infection of leucocytes, with detection of EA following infection of cells with low passage isolates of HCMV (Einhorn & Öst, 1984). Active infection of bone marrow precursor cells has also been demonstrated *in vitro*, although infection of the mature cells has not been demonstrated.

One may speculate that infection of PMNLs occurs solely during acute infection, perhaps following phagocytosis of infectious virions. Infection of monocytes and lymphocytes is a slower, more gradual process which may involve infection of precursor cells and the subsequent establishment of persistently infected clones and may lead to the establishment of latency.

It is virtually certain that the IE transcription detected represents a limited and unproductive infection rather than a low level productive infection, although transcription may be sufficient to facilitate production of IE antigen (Toorkey & Carrigan, 1989). Further viral transcription maybe prevented by control at the level of the RNA cascade in latent infection. Whether the limited transcripts detected in this study are similar to the latency associated transcripts (LATS) seen in HSV latency is not known. These LATS come from a specific, major early region of the HSV genome but are transcribed in the opposite (antisense) orientation and do not encode proteins. The HCMV transcripts appear to be of the correct polarity though the sequence and size of the complete transcripts have not been determined.

For true latency to arise in PBMC, infection of precursor cells must occur to enable the persistence of integrated HCMV DNA in the genome long after acute infection has resolved. The demonstration of latent
virus in such tissues as brain, kidney, spleen, lung, liver and arterial
wall (Toorkey & Carrigan, 1989; Hendrix et al, 1990) suggests a
multiplicity of sites from which virus may reactivate. In all these
cases, either IE RNA or IE antigen were detected but productive
infection was not demonstrated.
8.1 RESULTS

8.1.1 Initial Screening Results

First, a total of 6,789 donors were screened for the presence of HCMV-IgM using an EIA developed 'in house' and 513 (7.6%) were reactive. The specificity of the assay was intentionally set at a low level to ensure optimum sensitivity. The sensitivity was determined using a panel of 10 sera confirmed positive for varying titres of HCMV-IgM. All of these samples were consistently and clearly positive when tested using the assay.

8.1.2 Commercial Screening Results

Six of the 513 initially reactive donor samples were found to be repeatedly reactive using a commercial HCMV-IgM assay, (Captia M CMV IgM EIA, Mercia Diagnostics, Guildford UK) and were considered to be HCMV-IgM positive. The overall prevalence of HCMV-IgM in the donors studied was found to be six in 6,789 (0.09%). Assuming circulating IgM persists for an average of six months, the annual incidence of HCMV infection can be calculated as 0.18%.

8.1.3 HCMV-IgG Antibody Status

2,919 (43%) of the 6,789 donor samples initially screened for HCMV-IgM were found to be HCMV-IgG positive. All six of the HCMV-IgM positive samples identified were also found to be HCMV-IgG positive.
Table 8.1 Medical history of HCMV-IgM positive donors

<table>
<thead>
<tr>
<th>Donor Age</th>
<th>Sex</th>
<th>Medical Ab Titre</th>
<th>IgM</th>
<th>IgG</th>
<th>Total number</th>
<th>IgM-</th>
<th>Time since 1' donation</th>
<th>Ab Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>F none</td>
<td>1/16</td>
<td>1/32</td>
<td>2 neg</td>
<td>6/12</td>
<td>14/12</td>
<td>- 1/32</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>M none</td>
<td>1/4</td>
<td>1/16</td>
<td>1 pos</td>
<td>5/12</td>
<td>1/4 1/32</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>M none</td>
<td>1/16</td>
<td>1/64</td>
<td>0</td>
<td></td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>M none</td>
<td>1/2</td>
<td>1/8</td>
<td>1</td>
<td></td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>F none</td>
<td>1/4</td>
<td>1/16</td>
<td>0</td>
<td></td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>M none</td>
<td>1/8</td>
<td>1/8</td>
<td>0</td>
<td></td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

Thus six of 2,919 (0.2%) HCMV-IgG positive donors were also positive for HCMV-IgM. The titres of both HCMV-IgM and IgG in each of these six donors are presented in Table 8.1.

8.1.4 Rheumatoid Factor

The presence of rheumatoid factor could not be demonstrated in the sera of any of the six HCMV-IgM positive donors (Rheuma-Wellcotest, Wellcome Diagnostics, Dartford UK).

8.1.5 Donor Details

The age, sex, serostatus of subsequent donation(s), and any relevant medical history of the HCMV-IgM donors are also presented in table 8.1. Subsequently, to try to relate the HCMV-IgM status to the seroconversion data (Section 3.1.3), the previous donation history of the HCMV-IgM positive donors was also investigated (Table 8.2). Three donors were found to have given previous donations and of these two appeared to have seroconverted during the study period.
### Table 8.2 Previous donation history of HCMV-IgM positive donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age</th>
<th>Sex</th>
<th>Number</th>
<th>HCMV-Ab status</th>
<th>Isotype</th>
<th>Time before index donation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>F</td>
<td>0(N/D)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>M</td>
<td>1</td>
<td>neg</td>
<td>-</td>
<td>11/12</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>M</td>
<td>1</td>
<td>pos</td>
<td>n/k</td>
<td>6/12</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>M</td>
<td>2</td>
<td>neg/pos</td>
<td>n/k</td>
<td>15/12 6/12</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>F</td>
<td>0(N/D)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>M</td>
<td>2</td>
<td>n/k</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n/k = not known  
N/D = New donor

1 15/12 donation HCMV-Ab negative, 6/12 donation HCMV-IgG positive

The third donor had made only one previous donation, six months previously, and this was HCMV-IgG positive.

### 8.1.6 Dot-blot and PCR Analysis

Dot-blot analysis was performed on the serum and PBMC DNA from the HCMV-IgM positive donors with negative results in all cases. PCR analysis was also performed on the serum and PBMC DNA from the HCMV-IgM positive donors but HCMV DNA could not be detected in any of the serum samples. However, all six of the PBMC samples gave specific amplification products following DNA PCR (Plate 8.1), and three (50%) gave specific amplification products following RNA PCR (Plate 8.2). There was no apparent difference in the yield of product compared to DNA and RNA PCR of PBMC from HCMV-IgM negative, HCMV-IgG positive donors.
Plate 8.1  PCR analysis of PBMC DNA from HCMV-IgM positive donors. PCR products were electrophoresed on a 2% agarose/EtBr gel, blotted and probed with \(^{32}\text{P}\) labelled CMPR1. Lanes 1-6 PBMC DNA, lane 7- uninfected genomic DNA, lane 8- 1pg HCMV DNA. Numbers on the LH side represent DNA size markers (bp).
Plate 8.2  PCR analysis of PBMC RNA from HCMV-IgM positive donors. PCR products were electrophoresed on a 2\% agarose/EtBr gel, blotted and probed with $^{32}$P labelled CMPR2. Lanes 1-6 PBMC RNA, lane 7- uninfected cell RNA, lane 8- 1pg HCMV IE RNA. Numbers on the LH side represent DNA size markers (bp).
8.1.7 The IB Profile of HCMV-IgM Positive Donor Sera

The immunoblotting profiles of HCMV-IgM positive sera were similar to that seen with HCMV-IgG positive sera though fewer polypeptides were recognised. The response was primarily directed against polypeptides of apparent MWs 150, 55, and 40 kDa. Other polypeptides detected had apparent MWs of 115, 97, 88, 74, 66, 64, 60, 45 kDa (Plate 8.3). No polypeptides were recognised solely by HCMV-IgM. Insufficient samples were available to demonstrate any relationship between the polypeptides recognised and the antibody titre.
Plate 8.3  IB profiles of the HCMV-IgM positive donor sera. Strips 1-6 HCMV-IgM positive sera, strip 7- HCMV-IgM negative, -IgG positive serum. Numbers on the LH side represent approximate MWs (kDa).
HCMV-IgM was detected in 6 of 6789 (0.09%) blood donors, all of whom were also found to possess HCMV-IgG. This is the only estimate made in the UK and other reports are from the USA, where Beneke et al (1984) found HCMV-IgM in 7 of 529 (1.3%) donors, Preiksaitis et al (1985) in 1 of 235 (0.4%) and Jackson et al (1987) in 17 of 458 (3.7%); from Denmark, where Nielsen et al (1987) found HCMV-IgM in 1 of 314 (0.3%) and Wielaard et al (1986) found 1 of 600 (0.16%); and from Italy, where Musiani et al (1988) found HCMV-IgM in 6 of 55 (11%). Variation in the reported prevalence of HCMV-IgM may possibly be due to variability in the specificity of the assays used, although all the studies took account of factors known to cause false positive results in IgM assays. However, this variation is almost certainly due in part to differences in the prevalence of infections throughout the world. This is well illustrated by the finding of a wide ranging prevalence of HCMV-Ab within the USA; varying from 30-70% in different regions of the country (Tegtmeier, 1986).

None of the HCMV-IgM positive donors identified in this study had high titres of IgM Ab, the titres ranged from 1/2 - 1/16. The HCMV-IgG titres, however, were generally higher, ranging from 1/4 - 1/64. More detailed investigation of the immune response in these patients, using immunoblotting, showed that the HCMV-IgM response produced a well defined profile which recognised the expected polypeptides; likewise, the HCMV-IgG profile was similar to that found in HCMV-IgG positive, but -IgM negative donors.

Although it is generally accepted that, in blood donors, the presence of circulating HCMV-IgG is the most useful marker of previous
infection, some authors have proposed that other markers may be useful for identifying donors with a greater risk of transmitting HCMV. Donors with evidence of active or recent infection at the time of donation have been implicated in cases of post-transfusion HCMV infection (Beneke et al, 1984). A link between the presence of HCMV-IgM and an increased frequency of transmission has been reported by a number of workers (Tegtmeier, 1986), although this has not been confirmed in all such studies. Whilst the presence of HCMV-IgM usually indicates a recent or continuing primary infection, it has also been detected following episodes of reinfection (Chou et al, 1987a; Morris et al, 1990), though it is not clear whether the appearance of HCMV-IgM following reinfection indicates infectivity of asymptomatic immunocompetent individuals.

A further, more practical consideration regarding the prevalence and significance of HCMV-IgM in blood donors is that, in recent years, producers of commercial assays have begun to move from HCMV-total Ab assays to individual HCMV-isotype specific assays. These generally have greater sensitivity and specificity than the total Ab assays but could in theory lead to discrepant results, for example if a donor possessing only HCMV-IgM was tested using an 'HCMV-IgG specific' assay. However, such donors have not been found in this study.

The presence of HCMV-IgM in six donors in this study probably identifies recent infections with HCMV, although the length of time for which IgM persists following natural HCMV infection of healthy, immunocompetent individuals is poorly defined. The additional presence of HCMV-IgG suggests an early phase of convalescence. Discrimination between primary infection and reinfection is not possible in these cases. The annual incidence of primary infections, determined from the
prevalence of HCMV-IgM in the study population, was estimated to be 0.18%, a figure that is well below the observed seroconversion rate of 1.66% measured in the same study population. Using HCMV-IgM alone as a marker of recent infection would therefore result in identification of only about 10% of the donors seroconverting annually, i.e. those undergoing primary infection. If some of the HCMV-IgM positive donors were undergoing reinfection rather than primary infection, the detection of IgM would identify significantly less than 10% of primary infections.

Unfortunately, data providing information about the timing of infection in these HCMV-IgM donors was limited. Follow-up donations were obtained from only two of the six HCMV-IgM positive donors. Donor 1 (F, 21yr) gave two subsequent donations approximately 6 and 12 months after the index donation. Both of these donations were HCMV-IgM negative. The HCMV-IgG titres of the index donation and the subsequent donations were unchanged and therefore provide no information about the timing of infection in this donor. Donor 2 (M, 28yr), however, although providing only one subsequent donation, produced more interesting data. The subsequent donation was given approximately five months after the index donation and was also found to be HCMV-IgM positive, with the same Ab titre. Interestingly, the HCMV-IgG titre was found to have increased from 1/8 to 1/32. These findings suggest that, at the time of the index donation, this donor was either recovering from primary infection (at a time when the IgG response was still unfolding) or, alternatively, that he was recovering from a recent reinfection such that HCMV-IgM was present but the increase in HCMV-IgG titre, often seen following reinfection in immunocompromised individuals, had not yet occurred.
If, as has been proposed in at least one study (Beneke et al, 1984), there is an increase in the incidence of transmission of HCMV associated with HCMV-IgM positive donations, it appears likely that infectious or latent virus would be present in the donations and at a higher level than in IgG positive donations. However this was not found to be so. Free virus was not detected in the serum of these donors, and when equivalent quantities of PBMC DNA from HCMV-IgM positive and -IgG positive -IgM negative donors were analysed by PCR there was no apparent difference in the quantity of amplified product.

Dot-blot analysis of PBMC DNA from HCMV-IgM positive donors was also unsuccessful, a finding that agrees with the earlier findings of Jackson et al (1987). IE RNA was detected, by PCR, in PBMC from three of the six HCMV-IgM positive donors, and, as with DNA PCR, the quantity of product was similar to that obtained following PCR performed on RNA prepared from PBMC from HCMV-IgG positive and -IgM negative donors. The significance of detection of HCMV IE RNA in PBMC from healthy seropositive donors is discussed in detail in Section 7.2. However, the presence of IE RNA in the PBMC from HCMV-IgM positive donors may additionally result from productive infection.

Whilst direct comparison of symptomatic, clinical infections and those in healthy individuals may have limited value, the detection of free HCMV DNA in the serum of clinically infected individuals does at least demonstrate that a viraemic phase, albeit of limited duration, occurs during infection. The isolation of virus from PBMC is well documented and is performed in some laboratories as a routine diagnostic procedure.

Overall consideration of this data indicates that, in the donor population served by the N.E.Thames RTC, HCMV-IgM positive donations
present no greater risk of transmission than HCMV-IgG positive, -IgM negative donations. Furthermore, perhaps only 10% of the potentially more infectious donations may readily be identified. In contrast Beneke et al (1984) clearly demonstrated an increased incidence of infection associated with HCMV-IgM positive donations, though this study was performed in the USA where the incidence of infection is higher and was based upon data from donations from a mixed group of voluntary and paid donors. Such factors may account for the number of apparently infectious HCMV-IgM positive donations identified.
CHAPTER 9  PREVALENCE OF HCMV-IgA ANTIBODY

9.1 RESULTS

9.1.1 Prevalence of HCMV-IgA Antibody

Sera from a total of 500 selected donors, 350 seropositives (including the six HCMV-IgM positive donors) and 150 seronegatives, were analysed for the presence of HCMV-IgA. 10 of the 500 sera (2%) were found to contain HCMV-IgA, and two of these were also HCMV-IgM positive. Hence 33% of the HCMV-IgM positives were -IgA positive. All the HCMV-IgA positive donors possessed HCMV-IgG. The overall prevalence of HCMV-IgA in random blood donors was recalculated to be 1.5% (7.5 in 500), taking into account the bias in donor selection and the inclusion of the HCMV-IgM positive donors in the study population. None of the HCMV-IgA positive donors had any relevant medical history. The IgA status of any subsequent donations has not yet been determined (since, at the time of compilation of the data, insufficient time had elapsed since the index donation for the donors to be recalled).

Titration of the HCMV-IgA sera was not performed.

9.1.2 Dot-blot and PCR Analysis

None of the donors demonstrated any hybridisation, either with serum, or with PBMC DNA from the HCMV-IgA positive donors.

PCR analysis of the sera and PBMC from the eight HCMV-IgA positive donors that were HCMV-IgM negative gave similar results to those reported above (Section 8.1.6) for the HCMV-IgM positive donors. HCMV DNA was not detected in the serum of any donor. Seven of eight PBMC
DNA samples from the HCMV-IgA positive donors gave specific amplification products following DNA PCR in addition to the two IgM positive donors reported above (Plate 9.1). There was no apparent difference in the quantity of product compared with the amplification products from PBMC DNA of HCMV-IgA negative, -IgG positive donors. The PCR negative donor was repeatedly negative, although PCR analysis using the β-globin primers BEP1/BEP2 demonstrated that sufficient suitable DNA was present in the PCR reaction.

Five of the PBMC RNA samples gave specific amplification products following RNA PCR (Plate 9.2). When the results obtained from the two HCMV-IgM positive, -IgA positive donors are included in the overall results, HCMV RNA was detected in PBMC from five of the 10 donors (50%). IE RNA was not detected in either of the two donors found to be both HCMV-IgM and -IgA positive.

9.1.3 IB Profile of HCMV-IgA Positive Donor Sera

The IB response was directed against polypeptides with apparent MWs of 160, 150, 125, 82, 68, 55 and 45 kDa (Plate 9.3). Polypeptides, 150 and 125 kDa, were recognised by all of the HCMV-IgA sera. The sera did not give particularly strong reactions although the bands were clearly present. IB analysis of serum samples from the two HCMV-IgM, -IgA positive donors is shown in lanes 1 and 2 of Plate 9.3.
Plate 9.1  PCR analysis of PBMC DNA from HCMV-IgA positive donors. PCR products were electrophoresed on a 2% agarose/EtBr gel, blotted and probed with $^{32}$P labelled CMPRI. Lanes 1-8 PBMC DNA, lane 9- uninfected genomic DNA, lane 10- 1pg HCMV DNA. Numbers on the LH side represent DNA size markers (bp).
Plate 9.2 PCR analysis of PBMC RNA from HCMV-IgA positive donors. PCR products were electrophoresed on a 2% agarose/EtBr gel, blotted and probed with $^{32}$P labelled CMPR2. Lanes 1-8 PBMC RNA, lane 9- uninfected cell RNA, lane 10- 2pg HCMV IE RNA. Numbers on the LH side represent DNA size markers (bp).
Plate 9.3  IB profile of HCMV-IgA positive donor sera. Strips 1-10 HCMV-IgA positive donor sera, strip 11- HCMV-IgA positive control serum (from patient with acute HCMV infection) strip 12- HCMV-Ab negative serum. Numbers on the LH side represent approximate MWs (kDa).
9.2 DISCUSSION

Virus-specific IgA has been found following infection by a number of viruses but its role as part of the immune response to infection is not at all clear. HCMV-IgA has been found following both primary and recurrent infections of immunocompromised individuals, although limited data exists concerning the presence of HCMV-IgA in immunocompetent individuals. Interest in HCMV-IgA has primarily centered on determining the feasibility of using its presence to distinguish between primary and recurrent infection, particularly in allograft recipients. This study has considered the presence of HCMV-IgA in a similar way to that of HCMV-IgM, i.e. the prevalence and significance in immunocompetent individuals.

HCMV-IgA was detected in the sera of 10 of the 500 (2%) selected donors studied, an estimated prevalence of 1.5% for random blood donors. This figure is similar to that of 1.8% reported by Strand & Hoddevik (1984), but significantly lower than 18% (Sarov et al, 1982) or 80% (Doerr et al, 1987), all reported for various groups of immunocompetent individuals including blood donors. It is very clear that the dilution of serum and the type of assay used are of significance in the detection of HCMV-IgA. The 18% prevalence was determined by RIA (Sarov et al, 1982), whilst no positive sera were detected among the same samples by either of two enzymatic techniques. Similar variation has been reported by other authors. It is possible that the use of a less sensitive EIA technique explains the lower figure found in this study, even though a sensitive amplification system was incorporated into the assay. The IB results lend support to the EIA results as there were no discrepant results between the two techniques. If the EIA was too insensitive to detect some HCMV-
IgA positive sera, detection of Ab by IB in a number of EIA negative sera might have been expected. This situation did not occur, no discrepancies were detected between the EIA and IB results.

Whether the presence of HCMV-IgA indicates a greater infectivity of the donor is unknown. The presence of HCMV-IgA has been studied in patients with clinical HCMV infection and has been found potentially to be more useful as a marker of recurrent (reinfection) rather than primary infection, especially in the absence of HCMV-IgM, since the titre is boosted following recurrent infection (Doerr et al, 1987; Engelhard et al, 1991). Data concerning the persistence of HCMV-IgA is sparse but there is a consensus amongst the few published reports that HCMV-IgA persists for a slightly longer period than HCMV-IgM, and that both HCMV-IgM and -IgA precede HCMV-IgG.

In studies involving patients with proven clinical HCMV infection, great variation has been found in the persistence of HCMV-IgA, i.e. from as little as 30 days (Cranage et al, 1988; Engelhard et al, 1991; Strand & Hoddevik, 1984) to at least eight months (Levy & Sarov, 1980: Sarov et al, 1982). Unfortunately this study was completed before any of the HCMV-IgA donors were due for recall so that no data concerning the persistence of titre could be obtained. A more thorough investigation is required to determine the prevalence and persistence of HCMV-IgA in healthy donors.

As in the case of the HCMV-IgM positive donors identified, no specific evidence of either active or recent infection was found in the HCMV-IgA positive donors. The presence of HCMV-IgA in such individuals may simply reflect previous infection with HCMV, similar to the situation with HCMV-IgG. The possibility that HCMV-IgA marks
recurrent as well as primary infection in immunocompetent individuals also requires confirmation. The presence of both HCMV-IgA and -IgM in two donors may have greater significance and they may have been most recently infected with HCMV, and still at an early stage of their convalescence.

If HCMV-IgA persists longer after infection of immunocompetent individuals than HCMV-IgM, and also marks recurrent infection, it may be a more suitable marker of recent HCMV infection than HCMV-IgM, and screening of blood donors should therefore be considered. A problem is the lack of suitable commercial assays. Interestingly, HCMV-IgG was present in the sera of all of the -IgA positive donors so that (although they may mark more infectious donations) current screening regimens using isotype specific HCMV-Ab assays would not have failed to detect any of them.
CHAPTER 10 VIRURIA IN BLOOD DONORS

10.1 RESULTS

10.1.1 Study Group

A total of 276 donors and staff were approached and asked to provide urine samples. Of these, 258 were subsequently enlisted in the study, and 186 (72.1%), coded UP1-186, were HCMV-IgG positive and 72 (27.9%), coded UN1-72, were HCMV-IgG negative, at the time of entry.

10.1.2 Cell Culture

Satisfactory culture was performed on a total of 222 individuals, of whom 148 were HCMV-IgG positive and 54 were HCMV-IgG negative. Of the 258 samples provided for culture, 89 (34.5%) were initially, and 56 (21.7%) repeatedly toxic or contaminated and therefore unsuitable for culture. Virus was cultured from the urine of one (UP65) of the 148 successfully cultured seropositive individuals (0.61%), and none of the 54 seronegative individuals.

10.1.3 Dot-Blot Analysis

Dot-blot analysis was performed on DNA extracted from the pellets following ultracentrifugation of 200ml of clarified urine from each of the 258 donors. Analysis of samples UP1-180 was performed on the total amount of extracted DNA. The analysis of samples UP181-186 and UN1-72 was performed on 80% of the extracted DNA. None of the samples from the HCMV-IgG positive or negative individuals were found to contain detectable HCMV DNA. Dot-blot analysis of PBMC DNA from the viruric donor failed to detect any HCMV DNA.
10.1.4 DNA PCR Analysis

PCR analysis was performed on DNA extracted from all of the 258 urine samples. Because dot-blot analysis had already been performed on the total amount of DNA extracted from samples UP1-180, prior to the establishment of the PCR analysis technique, DNA for PCR analysis of samples UP1-180 was prepared from the stored duplicate urine samples. Analysis of samples UP181-186 and UN1-72 was performed on 20% of the DNA extracted from the pelleted urine.

One (UP65) of the 258 samples (0.39%) demonstrated a specific amplification product and was considered to be HCMV DNA positive (Plate 10.1). This sample was from urine of the donor found to be cell culture positive (Section 10.1.2). PCR analysis of the PBMC DNA from this donor was not performed as this work was performed prior to the introduction of PCR and a PBMC sample from this donor was unavailable for retrospective testing.

10.1.5 Medical History

The single viruric individual (UP65), a female aged 38 years, was healthy at the time of sample collection. Shortly after, however, she was investigated for a mild, glandular fever like illness. Subsequently, the diagnosis of glandular fever was not confirmed and no other diagnosis was made. The individual concerned was informed of these findings but chose not to inform her GP.

A further urine sample, collected approximately 1 year after the first, was not found to contain any detectable virus or viral DNA, by cell culture, or by dot-blot and PCR analyses.
Plate 10.1  PCR analysis of DNA extracted from donor urine samples. Lane 1- DNA size markers, lane 2- uninfected genomic DNA, lane 3- 1pg HCMV DNA, lanes 4-17 donor samples. A - PCR reaction products run on a 2% agarose/EtBr gel. B - S.blot of A probed with $^{32}$P labelled probe CMPR1. Numbers on the LH side represent DNA size markers (bp).
10.2 DISCUSSION

Diagnosis of acute HCMV infection is regularly made on the basis of clinical symptoms together with serological findings and isolation of virus, most commonly from urine. However, secretion of virus in the urine alone often does not correlate with virus-induced disease. Infection of some asymptomatic individuals may solely be marked by serology and urinary excretion of virus.

The detection of virus in the urine of one of the individuals studied was important but its significance is difficult to assess. The fact that the individual was in good health at the time of sample collection, and that no other markers of active or recent infection were apparent, may indicate persistent shedding of virus after an earlier infection. It is also possible that excretion of virus followed recurrent infection. However, as previous serum samples from the individual were not available, no rise in HCMV-IgG titre could be demonstrated and a distinction between primary infection and reinfection was therefore not possible. The fact that a further urine sample collected about 8 months after the positive sample was not found to contain virus or viral DNA provided little further significant information, except to demonstrate cessation of excretion within that period. Of interest, however, is that a short time after the index urine sample had been collected, the individual underwent a mild EBV-like illness which was investigated, though a complete diagnosis was not made. It appears very unlikely that HCMV viruria should precede symptomatic illness, since it usually develops later and often after the appearance of HCMV-Ab. The subsequent illness was therefore probably coincidental rather than significant.
Significantly, in one of the few accounts of urinary excretion of HCMV in blood donors, Toplin et al (1985), reported the persistence of HCMV in the urine of a donor for a period of at least one year. In this case, the persistence of virus enabled identification of an HCMV strain transmitted by transfusion to multiple recipients one year previously. In an earlier, specific study (Kane et al, 1975), virus was isolated from the urine of seven of 223 (3%) blood donors. All of these donors were HCMV-IgG positive but no other markers of active or recent infection were present and their HCMV-IgG titres did not distinguish them from the other donors. Virus was not detected in repeat urine samples from six of the donors taken 6-14 weeks later and there was no change in HCMV-IgG titre. Interestingly, each of three recipients of blood from three of the donors developed evidence of asymptomatic infection.

Excretion of virus in urine is common and often persists for long periods following an acute infection. Whether this necessarily represents a period during which donations may be infectious due to disseminated infection or is simply the result of continuing local productive infection in cells such as those lining the renal tubules is uncertain. The epithelial cells of the renal tubules are suitable sites for HCMV infection (Toorkey & Carrigan, 1988) and continuous or sporadic replication of virus may persist here in some individuals long after generalised infection has resolved, and resulting in the shedding of virus into the urine.
CHAPTER 11  EXCRETION OF HCMV IN SALIVA

11.1  RESULTS

11.1.1  Study Group

The group of donors asked to provide urine samples was also asked to provide a sample of saliva at the time of collection of the urine. Only 206 of the 276 donors subsequently provided saliva samples and entered this study. Of these, 133 (64.6%), coded SP1-133, were HCMV-IgG positive and 73 (35.4%), coded SN1-73, were HCMV-IgG negative.

11.1.2  Dot-blot and PCR Analysis

Dot-blot analysis was performed on 80% of the DNA extracted from the saliva from all 206 donors. None of the samples were found to contain detectable HCMV DNA. DNA PCR analysis was performed on the remaining 20% of the DNA extracted from the saliva of the 206 donors. None of the samples demonstrated specific amplification products.
11.2 DISCUSSION

Secretion of HCMV in saliva, often in the presence of secreted HCMV-Ab, is known to occur during acute infection. Apart from historical reports of the identification of a common 'cytomegalic agent' in the salivary glands of many stillborn foetuses and perinataly infected infant cadavers, few authors have studied secretion of virus in saliva.

Detection of virus in saliva has frequently been used in laboratory diagnosis of infection but is not necessarily associated with clinical disease. Salivary secretion may be a normal route of secretion of virus, the salivary glands certainly are sites of persistent infection, although whether latency may be established is not clear. It is possible that, like murine CMV, HCMV does not normally persist in the salivary glands for life, but may persist for extremely long periods in some individuals. The concomitant secretion of virus and HCMV-Ab in saliva demonstrates the limited efficacy of the humoral response in defence against HCMV and resolution of infection. The significance of any persistent infection is hard to gauge but presumably may result in the shedding of virus from apparently immune individuals, and may play a major role in the natural spread of infection to immunocompetent individuals. Salivary excretion of virus by blood donors may actually have little bearing on the potential infectivity of any donations. Donations given at this time should present no greater risk of transmission than donations from any HCMV-IgG positive donor. That the viruric donor did not secrete detectable virus demonstrates that the salivary gland and kidneys are likely to be independant sites of virus persistence.
Although many studies have examined post-transfusion HCMV infection, apart from detection of circulating HCMV-Ab, few have specifically studied the infectious state in blood donors to determine what other markers of potential infectivity may be present. This is perhaps understandable, since the simple procedure of screening donations for HCMV-Ab significantly reduces the risk of transmission. However, in countries with a high prevalence of HCMV-Ab, the provision of screened blood can cause major problems and other approaches require investigation. Such approaches aim either to reduce the risk of infection associated with each donation, for example by the removal of leucocytes by filtration, or to identify those donors who are more likely to transmit HCMV, for example by screening for HCMV-IgM Ab.

The advent of modern molecular and immunological techniques has not led only to increased sensitivity in conventional detection methods but also has facilitated more rapid laboratory diagnosis of infection. It is also possible to examine individual tissues for the presence of the nucleic acids of specific infectious agents rather than having to rely upon the appearance of specific antibody marking convalescence. Such techniques have been applied for a number of years to samples examined in clinical laboratories with remarkable success. As yet, they have not, however, been widely used in the field of Transfusion Microbiology. There are a number of reasons for this including the large number of samples to be examined, the cost, the need for a level of scientific support not widely available outside clinical research laboratories, and the fact that such complex techniques are neither suitable or necessary in many areas of Transfusion Microbiology. Some authors have, however, used such techniques to investigate further
specific results obtained during the routine screening of blood
donations (Harrison et al, 1985; Jackson et al, 1987). In particular, in
recent studies of hepatitis C virus (HCV), RNA PCR has been used to
demonstrate the presence of free virus in donor serum (Garson et al,
1990). Whilst such information may be of general interest, its
significance for transfusion practice is harder to assess.

Currently, patients at risk of HCMV infection receive blood screened
for the presence of HCMV-Ab, usually using an EIA specific for HCMV-
IgG. Although it cannot be assumed that the assays used have
absolute sensitivity, such screening appears to be effective, the
transfusion of HCMV-Ab negative blood to a seronegative recipient
rarely leads to PT HCMV infection. This compares to a risk of 0.38%
per unit when HCMV-Ab positive blood is transfused to an HCMV-Ab
negative patient (Preiksaitis et al, 1988a). Unfortunately, data on
transmission are limited and there are no data currently available
regarding post transfusion HCMV infection in the UK; the effectiveness
of current HCMV-Ab screening programmes cannot therefore be
monitored. In the N.E.Thames Region, no cases of transmission of HCMV
from donors screened as HCMV-Ab negative have been reported.

It is clear that not all HCMV-Ab positive donations transmit HCMV,
even when transfused to a susceptible recipient. Evidently, a number
of factors, specific to both donor and recipient, are involved in the
acquisition of PT HCMV. Some studies have been performed to try to
identify donors who are more likely to transmit HCMV. These have
involved the detection of markers of infection, other than HCMV-IgG,
that were thought to indicate recent HCMV infection and a possible
risk of infectious virus still being present in the donated blood.
Accordingly, the presence of HCMV-IgM, potentially marking both
primary and secondary (recurrence/reinfection) infection has been studied. Whilst initially the presence of HCMV-IgM was shown to be associated with an increased incidence of PT HCMV infection (Beneke et al, 1984), subsequently other authors have not found this to be the case (Preiksaitis et al, 1985; Wilhelm et al, 1986).

To date, the presence of HCMV-IgA has not been used to mark potentially infectious donations in the same manner as HCMV-IgM. HCMV-IgA appears after both primary and secondary infections and has been considered to be a possible specific marker of secondary infection in immunocompromised individuals. Interestingly, when the individual prevalence figures for HCMV-IgM and -IgA are combined, the figure obtained (3.18%), which may be considered to represent the annual incidence of HCMV infection in the group of donors studied, is similar to the expected mean seroconversion rate in the same group of donors (2.56%). Whether the similarity between the combined prevalences of HCMV-IgM and -IgA and the observed seroconversion rate is coincidental or significant is hard to determine. However, if markers of potentially more infectious donations are earnestly being sought then, on the basis of these results, the screening of donors for both HCMV-IgM and -IgA should in theory detect all recently infected (primary or secondary), and therefore potentially more infectious, donors. The incidence of secondary infections in immunocompetent individuals is unknown as it is virtually impossible, under normal circumstances, to identify such donors. A possible approach would be the identification of HCMV-IgM and/or -IgA in previously HCMV-IgG positive donors, or of a significant rise in titre of the existing HCMV-IgG. Although it is interesting to speculate whether the small number of HCMV-Ab positive donors who transmit
HCMV comprises those donors recently infected with HCMV, this study has failed to identify any increased risk associated with such donors.

The results obtained using molecular techniques, when considered together with the limited published data, have provided a firm basis for the identification of the PBMC as a site of latency of HCMV. The demonstration of HCMV NA in PBMC of HCMV-Ab negative individuals is a potentially important finding. Furthermore, it is important to determine whether the transfusion of blood from these donors does give rise to PT HCMV infection. Although no reports of transmission of HCMV from HCMV-Ab negative donations have yet been received, there are a number of reasons why transmission has not been seen. Firstly, if infection is asymptomatic, it is very unlikely that it will be diagnosed. Secondly, only a small percentage of HCMV-Ab negative donations have been found to contain HCMV NA. Thirdly, only a small percentage of susceptible recipients will actually acquire PT infection.

However, it is clear that this study, together with the previous studies of Schrier et al (1985) and Nelson et al (1990), have demonstrated the presence of a previously unidentified population of donors in whom potentially infectious NA is present without detectable circulating HCMV-specific Ab (PCR positive/HCMV-Ab negative). Although a number of possible explanations for these findings have been proposed, it seems probable that the most reasonable explanation is that in many cases HCMV-Ab is present, but at a level below that detectable using the currently available HCMV-Ab screening assays.

The problem then arises as to how to identify such donors in a routine screening situation. Identification would either rely upon the detection of viral NA or would require an increase in the sensitivity
of HCMV-Ab detection techniques to enable detection of the low levels of antibody possibly present in such individuals. Although molecular techniques would identify the NA in these donors, their use is not appropriate in this situation as the techniques are not at a stage where they can be applied to mass screening situations. The detection of HCMV-Ab must therefore remain as the most suitable approach, at least for the foreseeable future. However, to detect the apparently low levels of antibody that may exist, a significant increase in the sensitivity of such assays without any concomitant loss of specificity is required. It would then be possible to use the PCR positive/HCMV-Ab negative donor samples identified in this study as controls in the development and evaluation of these more sensitive HCMV-Ab assays.

The recently developed RIST assay (Klapper et al, 1990) has already been discussed briefly and it is quite possible that it represents the first of a new generation of more sensitive assays. Thus, although when compared with the currently available commercial HCMV-Ab assays the RIST assay appears to suffer from lack of specificity, perhaps it should now be considered that the commercial assays actually suffer from a lack of sensitivity; the RIST assay being able to detect very low levels of HCMV-Ab in samples that appeared HCMV-Ab negative with standard commercial HCMV-Ab assays.

In conclusion, although the use of molecular techniques has enabled the identification of a previously unidentifiable population of donors who potentially may transmit HCMV, at the present time such techniques are not practical for the screening of blood donors. The future routine detection of such donors must rely on conventional serology but using HCMV-Ab screening assays with a greater sensitivity that that routinely achieved at present.
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Preparation of Solutions and Reagents

All reagents and chemicals used were high grade; sterile distilled water was used to prepare solutions; all glass- and plasticware was thoroughly cleaned in Decon 90, rinsed and if suitable, autoclaved prior to use. All suitable solutions were autoclaved prior to use.

All solutions used for RNA work were either prepared and then treated with 0·1% DEPC, or in the case of Tris and MOPS solutions, were prepared with DEPC treated water.

(i) Enzyme Immunoassay Reagents

Phosphate buffered saline (PBSA)
  Dulbecco's solution A (Oxoid)

Sample diluent
  PBSA, 2% goat serum, 0·1% tween 20.

Wash buffer
  PBSA, 0·1% tween 20, 0·1% Bronidox.

0·05M Bicarbonate coating buffer (pH 9·6)
  1·59gm sodium carbonate
  2·93gm sodium hydrogen carbonate
  Dissolved in 1 litre of distilled water and stored at 4°C

0·1M Citrate buffer
  44·1gm tri-sodium citrate
  500μl hydrogen peroxide
  Dissolved in 1 litre of distilled water and stored at 4°C.

3 3'D5'Tetramethylbenzidine (TMB) substrate
  One 1mg TMB tablet (Sigma) dissolved in 5ml of distilled water. 5ml
  of 0·1M citrate buffer added and 100μl immediately added to each
  well.

IQ Bio Substrate/Amplifier system
  Details of the contents of this system are not generally available
  due to licensing agreements.
  Substrate solution - NADH
  Amplifier solution - alcohol, alcohol dehydrogenase, diaphorase,
    iodonitrotetrazolium violet
  Wash buffer - piperazine buffer pH 10·5

(ii) Immunoblotting Reagents

Acrylamide (Bis/acrylamide)
  Prepared as a 40% solution.
  40gm acrylamide
  1·08gm N,N’-methylenebisacrylamide
  Made up to 100ml with distilled water, filtered through Whatman No.
  1 paper and stored in the dark at 4°C for up to one month.
Acrylamide Gels
9% resolving gel (10ml)
5ml 2x resolving gel buffer
2.55ml distilled water
2.25ml 40% bis/acrylamide
200μl 2% ammonium persulphate
10μl TEMED
Solution degassed prior to the addition of ammonium persulphate and TEMED

4% stacking gel (10ml)
5ml 2x stacking gel buffer
3.75ml distilled water
1ml bis/acrylamide
250μl 2% ammonium persulphate
10μl TEMED
Solution degassed prior to the addition of ammonium persulphate and TEMED

Ammonium persulphate (2%)
0.2gm ammonium persulphate.
Dissolved in 10ml of distilled water. Stored at 4°C for up to one week.

Antibody buffer (10ml)
1ml TBTS (10x)
200μl goat serum
Made up to 10ml with distilled water. Made fresh when required.

Blocking solution (10ml)
10ml PBSA
200μl goat serum
0.5gm skimmed milk powder (Marvel™)
Made up to 10ml with distilled water. Made fresh when required.

Cell lysis (sample) buffer (2x)
4gm SDS
20gm glycerol
2ml 0.1% bromophenolblue
2ml 2-mercaptoethanol
50ml 2x stacking gel buffer
Made up to 100ml with distilled water.

Chloro-naphthol substrate solution
30mg of 4-chloro-1-naphthol (BIO-RAD) dissolved in 10ml of ice cold methanol. 30μl of ice cold hydrogen peroxide added to 50ml of TBS, the chloro-naphthol solution added and the solution used immediately.

Gel destain
50ml acetic acid
100ml methanol
Made up to 500ml with distilled water.
Gel stain
50ml acetic acid
250ml methanol
10mg coomassie blue
Made up to 500ml with distilled water.

NNN'-N-tetramethylenediamine (TEMED)
Used as supplied. Stored at room temperature.

Reaction buffer (10ml)
1ml TBTS (10x)
200ul goat serum
0.5gm skimmed milk powder (Marvel™)
Made up to 10ml with distilled water. Made fresh when required.

Resolving gel buffer (2x)
90.6gm tris base
2gm SDS
Made up to 900ml with distilled water, adjusted to pH 8.8 with conc. HCl and made up to 1 litre with distilled water. Stored at room temperature.

Running buffer (5x)
45gm tris base
15gm SDS
216gm glycine
Made up to 3 litres with distilled water, pH checked as approximately pH 8.3 (DO NOT ADJUST), stored at 4°C.

Stacking gel buffer (2x)
30.24gm tris base
2gm SDS
Made up to 900ml with distilled water, adjusted to pH 6.8 with conc. HCl and made up to 1 litre with distilled water, stored at room temperature.

Transfer buffer (10x)
30.3gm tris base
144gm glycine
1.5gm SDS
Made up to 1 litre with distilled water, stored at 4°C.

Tris buffered saline (TBS)
20mM tris.HCl pH 7.5
500mM sodium chloride

Tris buffered tween saline (TBTS) (10x)
100mM tris.HCl pH 7.5
150mM sodium chloride
0.1% tween 20
(iii) Molecular Biology Reagents

Blu Gene blocking buffer
0.1M tris.HCl pH 7.5
0.15M sodium chloride
3% BSA (fract. V)
Made fresh when required

Blu Gene stop solution
20mM tris.HCl pH 7.5
0.5mM EDTA

Blu Gene substrate buffer
0.1M tris.HCl pH 9.5
0.1M sodium chloride
50mM magnesium chloride

Blu Gene substrate solution
33μl 75mg/ml nitroblue tetrazolium (NBT) in dimethylformamide
25μl 50mg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) in dimethylformamide
Added to 7.5ml of wash buffer and used immediately.

Blu Gene wash buffer
0.1M tris.HCl pH 7.5
0.15M sodium chloride

DEAE-Sephacel
DEAE-Sephacel (Pharmacia) equilibrated in TE pH 7.6, 0.6M NaCl.

Deionised formamide
100ml formamide
5gm AG 501-X8 mixed bed ion exchange resin (Bio-Rad)
Mixed and stirred for 30 minutes at room temperature. Filtered twice through Whatman No. 1 paper. Stored at 4°C.

Denhardt's solution (50x)
5gm ficoll
5gm polyvinylpyrrolidone
5gm bovine serum albumin (Fraction V)
Dissolved in 500ml of distilled water. Stored at 4°C.

Ethylenediaminetetraacetic acid (EDTA) (0.5M)
186.1gm di-sodium EDTA
Dissolved in 800ml of distilled water, pH adjusted to 8.0 and made up to 1L.

Ficoll/Triosil gradient
Six ampoules of Triosil 440 dissolved in 264ml of distilled water, made up to 900ml with 9% ficoll 400 solution (Pharmacia). Specific gravity checked as 1.078. Autoclaved and stored at room temperature.
Guanidinium thiocyanate denaturing solution
250gm guanidinium thiocyanate (Fluka)
0.75M sodium citrate
10% Sarcosyl
293ml of distilled water added to the thiocyanate in the manufacturers container followed by 17.6ml of sodium citrate and 26.4ml of sarcosyl heated to 65°C. Stored at room temperature for 3 months. Denaturing solution prepared by adding 0.36ml β-2ME/50ml of above stock solution.

IPTG (Isopropylthio-β-D-galactoside)
2gm IPTG
Dissolved in 8ml of distilled water, then made up to 10ml. Sterile filtered and stored in 1ml aliquots at -20°C.

LB (Luria-Bertani) Medium
10gm Bacto-tryptone
5gm Bacto-yeast extract
10gm sodium chloride
Dissolved in 950ml of distilled water, pH adjusted to pH 7 and made up to 1 litre. Sterilised by autoclaving.

MOPS Buffer (10x)
200mM MOPS (3-(N-Morpholino)propane-sulphonic acid)
50mM Sodium acetate pH 7
10mM EDTA

PEG (polyethylene glycol) (100ml)
20gm PEG 8000
14.6gm sodium chloride
Dissolved in 90ml of distilled water and made up to 100ml. Sterilised by filtration and stored at room temperature.

Saline tris EDTA (STE)
10mM tris.HCl pH 8.0
100mM sodium chloride
1mM EDTA

Sephadex G-50
Added to distilled water, washed and equilibrated in TE pH 8. Stored at 4°C with 0.1% Na azide.

Sodium acetate (3M)
408.1gm sodium acetate
Dissolved in 800ml of distilled water, pH adjusted to 5.2 with glacial acetic acid and made up to 1L.

Sodium dodecyl sulphate (SDS) (10%)
100gm sodium dodecyl sulphate
Dissolved in 800ml of distilled water, pH adjusted to 7.2, and made up to 1L.

Standard saline citrate (20x SSC)
175.3gm sodium chloride
88.2gm sodium citrate
Dissolved in 1L of distilled water, pH adjusted to 7.0.
Standard saline phosphate EDTA (20x SSPE)
174gm sodium chloride
27.6gm sodium dihydrogen orthophosphate
7.4gm EDTA
Dissolved in 800ml of distilled water, pH adjusted to 7.4 and made up to 1L.

TAE (tris-acetate) buffer (50x)
242gm tris base
57.1ml glacial acetic acid
100ml 0.5M EDTA
Dissolved in 900ml of distilled water and made up to 1 litre.

TBE (tris-borate) buffer (10x)
109gm tris base
55gm boric acid
40ml 0.5M EDTA
Dissolved in 900ml of distilled water and made up to 1 litre.

TG-2 cells
A derivative of JM101 cell line suitable for optimal transfection. Stored plated onto minimal medium and picked into LB medium for use.

Tris EDTA (TE)
\[ \text{pH 8.0} \]
10mM Tris.HCl pH 8.0
1mM EDTA
\[ \text{pH 7.6} \]
10mM Tris.HCl pH 7.6
1mM EDTA

Urea/acrylamide gel (70ml)
7ml TBE (10x)
10.5ml acrylamide (40% acrylamide, 19:1 acrylamide/bis)
29.4gm urea
420μl 25% ammonium persulphate
70μl TEMED
All except ammonium persulphate and TEMED dissolved in 25ml of distilled water. Volume adjusted to 70ml with distilled water, persulphate and TEMED added.

X-GAL (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)
Dissolved in dimethylformamide to a concentration of 20mg/ml. Wrapped in foil and stored at -20°C.

YT Medium
10gm bacto-tryptone
10gm bacto-yeast extract
5gm sodium chloride
Dissolved in 900ml of distilled water. pH adjusted to pH 7.0 with 5N sodium hydroxide solution. Made up to 1 litre and autoclaved.
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