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A STUDY ON THE CELL MEDIATED IMMUNITY OF HUMAN CYTOMEGALOVIRUS INFECTION IN KIDNEY TRANSPLANT RECIPIENTS

BY

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DECLARATION

All work reported in this thesis was performed by the author in the laboratory of Dr Alexander Pacsa at the Department of Microbiology, Faculty of Medicine, Kuwait University.

Three papers were published from this work, reprints attached:

1. **Essa, S., Raghupathy, R., Pacsa, A., El-Shazly, A., Tareq, S., Azizieh, F.** (2000). Th1-type cytokines production is decreased in kidney transplant recipients with active CMV infection. *J. Med. Virol.* 60, 223-229.

2. **Essa, S., Pacsa, A., Al-Attiya, R., El-Shazly, A., Raghupathy, R., Tareq, S (2000).** The use of flow cytometer for the detection of CMV specific antigen (pp65) in leukocytes of kidney recipients. *Clin. Transpl.* **14**, 147-151 .

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Work described in this thesis has not been submitted for a degree at any other institute.

SUMMARY

Cytomegalovirus (CMV) infection is a major complication after kidney transplantation. Despite antiviral therapy it contributes significantly to high morbidity. This study was aimed at (a) detecting a CMV specific antigen pp65 in CMV-infected fibroblast cells and in leukocytes of kidney transplant recipients by flow cytometric assay (FCA) (b) determining the stimulation index (S.I.) of phytohaemagglutinin (PHA) and CMV-stimulated peripheral blood mononuclear cells (PBMC), (c) determining the levels of Th1- and Th-2 related cytokines in the supernatant of stimulated PBMC from kidney transplant recipients with and without active CMV infection (d) determining immunophenotyping of cells found in the peripheral blood of CMV-infected and CMV-uninfected kidney transplant recipients by flow cytometry using antibodies specific to $CD2^+$ (pan T), $CD3^+$ (mature T), $CD4^+$ (T helper), $CD8^+$ (T suppressor), $CD26^+$(T activated), $CD16^+/CD56^+$ (NK cell), $CD19^+$(pan B), $CD15^+$ (granulocytes). Thirty-five patients with, and 44 without active CMV infections, as diagnosed by a CMV antigenemia assay (AA), were inducted into this study. FCA distinguished clearly between the infected and uninfected fibroblast cells. Regarding kidney transplant recipients, the FCA was positive when the number of AA positive cells was five or more per 5×10^4. Moreover, the percentage of antigenemia-positive cells by FCA correlated well with symptomatic CMV infections. After PHA and CMV stimulation of PBMC from patients, S.I. was determined by radioactive thymidine uptake while the production of Th1-type cytokines [interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α)] and Th2-type cytokines (IL-4, IL-10) were measured by ELISA. PBMC of patients with active CMV infection showed significantly lower S.I. values than patients without an ongoing CMV infection ($p<0.0001$). Levels of Th2-type cytokines in CMV-infected and uninfected kidney recipients were similar; however, the levels of the Th1-type cytokines were significantly lower in CMV-infected patients ($p<0.05$). Low levels of Th1-type cytokines seem to correlate well with active CMV infection in kidney recipients. The percentage of $CD3^+$ immunocompetent T lymphocytes and $CD4^+$ T lymphocytes were consistently higher in kidney transplant recipients without an active CMV infection than in the group of recipients with an active CMV infection. These differences were statistically significant in the case of $CD3^+$ ($p<0.05$) and $CD4^+$ ($p<0.005$). On the other hand the difference in percentage $CD2^+$, $CD8^+$, $CD16^+$+$CD56^+$, $CD19^+$, $CD15^+$ cells were statistically insignificant. Therefore, these data suggest that active CMV infection in kidney transplant recipients is associated with a significant alteration in the lymphocyte proliferative responses, the levels of Th1-type cytokines (IFN-γ, TNF-α, IL-2), and the percentage of $CD3^+$, $CD4^+$ when compared to kidney transplant recipients without active CMV infection.

ABBREVIATIONS

AA	antigenemia assay
ACV	acyclovir
AIDS	acquired immunodeficiency syndrome
AP	assembly protein
ATCC	American type culture collection
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BMT	bone marrow transplant
CD	cluster of differentiation
CID	cytomegalic inclusion disease
CMV	cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocytes
D	dalton
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
E	early
EBV	Epstein-Bar virus
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FBS	fetal bovine serum
FCA	flow cytometric assay
FI	fluorescence intensity
FITC	fluorescein isothiocyanide
g	glycoprotein
GCV	gancyclovir
GM-CSF	granulocytes macrophage colony stimulating factor
HC	heavy chain
HHV	human herpes virus
HIV	human immunodeficiency virus
HLA	human lymphocyte antigen
HSV-1	herpes simplex virus type-1
HSV-2	herpes simplex virus type-2
ICTV	International Committee on the Taxonomy of Viruses
IE	immediate early
IFN	interferon
IL	interleukin
IR_L	internal long repeat
IR_S	internal short repeat

kbp	kilobase pair
kD	kilodalton
L	late
MCMV	murine CMV
MCP	major capsid protein
mCP	minor capsid protein
MEM	minimal essential medium
MHC	major histocompatibility complex
NIEPs	noninfectious enveloped particles
NK	natural killer cells
nm	nanometre
p	probability
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PMNL	polymorphonuclear leukocytes
Pp	phosphoprotein
RCMV	rat CMV
Rsq	Regression square
SCP	smallest capsid protein
SD	standard deviation
SEM	standard error of mean
S.I.	stimulation index
TAP	transporters associated with antigen processing
TCID	tissue culture infectious dose
Th	T helper
TGF	transforming growth factor
TNF	tumor necrosis factor
TR_L	terminal long repeat
TR_S	terminal short repeat
T/V	trypsin/versene
VZV	varicella-zoster virus
MEOH	methanol
MW	molecular weight
UL	unique long
US	unique short

1. INTRODUCTION

1.1 Herpesviruses.

The herpes family contains several of the most important human pathogens. Clinically, the herpesviruses exhibit a spectrum of diseases. Some have a wide host-cell range, whereas others have a narrow host-cell range. The outstanding property of herpesviruses is their ability to establish lifelong persistent infections in their hosts and to undergo periodic reactivation. Their frequent reactivation in immunosuppressed patients causes serious health complications. Curiously, the reactivated infection may be clinically quite different from the disease caused by the primary infection. Herpesviruses posses a large number of genes, some of which have proved to play an important part in pathogenesis.

Eight herpesviruses commonly infect humans: herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpesvirus types 6 and 7 (HHV6 and HHV7). There are nearly 100 viruses of the herpes group that infect many different animal species. The existence of a new human herpesvirus type 8 (HHV8) has been reported (Moore and Chang, 1995).

1.1.1 Properties of herpesviruses.

Important properties of herpesviruses are summarized in Table 1.

Table 1. Important properties of herpesviruses

Virion: Spherical, 120-300 nm in diameter (icosahedral capsid, 100 nm).
Genome: Double-stranded DNA, linear, MW 95-150 million, 120-240 kbp, reiterated sequences.
Proteins: More than 35 proteins in virion.
Envelope: Contains viral glycoproteins, Fc receptors.
Replication: Nucleus, bud from nuclear membrane.
Outstanding characteristics: Establish latent infections; persist indefinitely in infected hosts, are frequently reactivated in immunosuppressed hosts.

1.1.2 Structure and Compositions.

Herpesviruses are large viruses. Different members of the group share architectural details and are indistinguishable by electron microscopy. All herpesviruses have a core of double-stranded DNA, in the form of a torus (Nazerian, 1974), surrounded by a protein coat that exhibits icosahedral symmetry and has 162 capsomeres (Wildy and Watson, 1963). The nucleocapsid is surrounded by an envelope that is derived from the nuclear membrane of the infected cell and contains viral glycoprotein spikes approximately 8 nm in length (Wildy and Watson, 1963) (Fig. 1).

The tegument, a term introduced by Roizman and Furlong (1974) to describe the structures between the capsid and envelope, has no distinctive features in thin sections but may appear to be fibrous on negative staining (Furlong *et al.*, 1972; Wildy and Watson, 1963). The tegument is frequently distributed asymmetrically.

The size of herpesvirions has been reported to vary from 120 to nearly 300 nm (Roizman and Furlong, 1974). The variation is in part due to variability in the thickness of tegument. Another major source of variability is the state of the envelope. The double-stranded DNA genome (MW 95-150$\times 10^6$; 120-240 kbp) is linear and circularizes immediately upon release from capsids into the nuclei of infected cells.

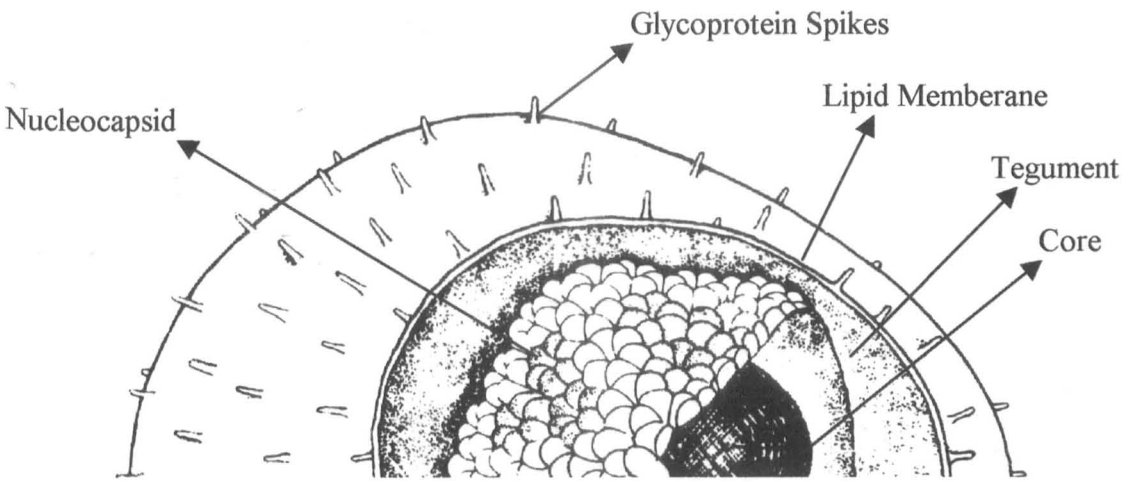


Figure 1. Diagram of herpesvirus and its nucleocapsid. Modified virsion from Atlas of virus diagram by Hans-W Ackermann and Laurent Berthiaume.

1.1.3 Classification.

Herpes viruses share various biological properties. For example, synthesis of viral DNA and assembly of the capsids occurs in the nucleus of an infected cell, and host virus production is accompanied by the destruction of the cell. The DNA of the herpes viruses may also remain latently present in their host cells. In such latently infected cells, the viral genomes take the form of closed circular molecules. Classification of the numerous members of the herpesvirus family is complicated. A useful division into subfamilies is based on biological properties of the agents. The members of the family *Herpesviridae* have been classified by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses (ICTV) into three subfamilies (i.e., the *alphaherpesvirnae*, the *betaherpesvirinae* and the *gammaherpesvirinae*) on the basis of biological properties. *Alphaherpesvirinae* are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia. This subfamily contains the genera *Simplexvirus* (HSV-1, HSV-2) and *Varicellovirus* (VZV). The subfamily *betaherpesvirinae* is characterised by a restricted host range. The reproductive cycle is long and the infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegalia). The virus can be maintained in latent form in sensory ganglia, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera CMV, and HHV6. In line with this, the nucleotide sequences of the HHV-6 genome is more closely related to that of CMV than to that of the other herpesviruses. This is also reflected at the protein level, where there are several closely related proteins, for example the glycoprotein B, the major capsid protein (MCP), and the DNA binding protein (p52). The target cells of *gammaherpesvirinea* like EBV are lymphoblastoid cells, and some cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are specific for T- or B-lymphocytes. Latent virus is frequently demonstrated in lymphoid tissue. This subfamily contains the genera *Lymphocryptovirus* (EBV).

1.2 Human cytomegalovirus.

5-10% of adult population throughout the world escape infection with CMV in Kuwait more than 90% are infected. The presence and type of clinical manifestations, and the sites and extent of virus excretion vary, but serological surveys conducted on all continents confirm the ubiquitous distribution of CMV (Weller, 1971).

CMV characteristically produces cell enlargement with intranuclear inclusions. These cellular changes seen in tissues of patients with fatal infection led to the early designation of the term "cytomegalic inclusion disease" (CID), even before the causative agent was isolated (Hanshaw, 1968). Humans are believed to be the only reservoir for CMV, so CMV is highly species-specific and can be propagated *in vitro* in cells of human origin (human fibroblastic tissue). CMV, including the commonly studied laboratory strain AD169, was first isolated in human fibroblast cell culture independently in three laboratories (Rowe *et al.*, 1956; Smith, 1956; Weller *et al.*, 1957). Strains of AD169 are widely used in many laboratories, adapted to grow well in laboratory conditions. Clinical isolates of CMV exhibit different levels of virulence and tissue tropism. This might be attributed to the fact that fresh clinical isolates carry at least 19 genes that are not found in laborarory strains (Cha *et al.*, 1996). CMV replicate in human fibroblast cell cultures very slowly resulting in a small amount of cell-free virus. Infection usually spreads from cell to cell. Consequently, it may take sometimes several weeks for the development of observable cytopathic effect. Though many genetically different CMV strains circulate in the human population, these strains show a high degree of antigen similarity.

The CMV genome is the largest of all known human herpes viruses. It contains more than 235 kbp encoding for at least 200 proteins (Chee *et al.*, 1990). The genome has a high GC content (57.2%) and is divided into a unique long (UL) and a unique short (US) region, each of which is flanked by an internal (IR_L, IR_S) and terminal (TR_L, TR_S) base pair repeat sequences (Fig. 2).

Figure 2. Structural organization of the human CMV genome. U_L and U_S represent the long and short unique sequences, respectively. Terminal (TR) and internal repeat (IR) sequences are indicated by black boxes

Three types of virus particles are produced in the CMV-infected cell. Firstly, the characteristic infectious virion. Secondly, particles that resemble the original virion in morphology but lack DNA, the noninfectious enveloped particles (NIEPs). Thirdly, the so-called dense bodies which lack both nucleocapsid and viral DNA. Dense bodies which are largely composed of the tegument protein pp65 usually accumulate in the cytoplasm of infected cells and surronded by an envelope containing glycoproteins (Irmiere and Gibson, 1983; Klages *et al.*, 1989; Landini *et al.*, 1987). *In vitro*, virions, NIEPs, and dense bodies all have the same capacity to penetrate the host cell. Immediately after the viral protein fuses with the cell membrane the capsid surrounded by the matrix enters the cytoplasm (Topilko and Michelson, 1994).

Similarly to other herpesviruses, CMV replication is tightly regulated by a multistep process. During a productive infection, cellular transcription factors probably interact with a long regulatory viral DNA sequence and initiate the transcription of a small number of immediate early (IE) genes which are believed to be the key regulators controling viral replication. At least some of the IE proteins represent nonstructural regulatory proteins which induce the expression of early (E) genes. Early (E) proteins are required for viral DNA replication. Following viral DNA replication, late (L) proteins are synthesized, many of which are incorporated into the virion or aid the process of progeny assembly.

The capsid of CMV consists of a small number of proteins. Immature capsids, the so called B-capsids, which are particles that still need to be packed with DNA and enveloped, are largely composed of the following proteins: the MCP, the assembly protein (AP), the minor capsid protein (mCP), and the smallest capsid protein (SCP). It is postulated that the assembly protein plays a role in DNA packaging. Immature capsids can be enveloped to produce NIEPs or be filled with DNA and subsequently enveloped to become virions.

Several proteins can be found in the tegument. However, the most abundant tegument proteins are the basic phoshoprotein pp150, a product of UL32, and the lower matrix protein pp65, a product of UL83. The function of both proteins is not clear yet, but comparison with known functions of similar proteins in other herpes

viruses suggests that they are involved in viral gene regulation and in modification of the host cell metabolism (Mocarski, 1993).

The lower matrix protein pp65 is the most abundantly produced tegument component of CMV in infected fibroblasts. In dense bodies pp65 accumulates to generate up to 95% of the protein mass. Nevertheless, pp65 is hardly present in virions (Hensel *et al.*, 1995; Irmiere and Gibson, 1983). During active CMV infection, accumulation of pp65 can be detected in peripheral blood leukocytes (Grefte *et al.*, 1992a,b). This technique, known as the CMV-antigenemia assay (AA), can be used as a valuable diagnostic tool to demonstrate and quantify CMV infection. Two studies tried to define the importance of the gene encoding the pp65 in the virus cycle. In one study, in which a pp65 deletion mutant virus was used, it appeared to be a non-essential protein for viral replication *in vitro* (Schmolke *et al.*, 1995b). However, in another study, which used a stably expressed antisense RNA to the UL83 gene which encodes for pp65, viral replication was severely inhibited (Dal Monte *et al.*, 1996). More work is required to resolve these apparent differences.

Of the three major viral phosphoproteins pp65, pp150, and pp71, pp71 is the minor component. Pp71 is also known as the upper matrix protein and is the product of the UL82 gene, immediately adjacent to the pp65 gene. Both proteins, pp71 and pp65, are translocated to the nucleus of the cell during the first minutes after virus uptake by a cell (Liu and Stinski, 1992; Schmolke *et al.*, 1995a). Two other CMV tegument phosphoproteins have been identified, but their function has not been elucidated yet: pp28, which is the highly immunogenic product of the UL99 gene, and pp67, a product from a spliced transcript that includes a portion of the UL65 gene.

To date, three distinct families of glycoprotein complexes have been described to be present in the CMV envelope; gCI, gCII, and gCIII. The gCI complex, better known as the gB homologue of HSV (CMV gB) is the most studied CMV induced glycoprotein. gCI, encoded by the UL55 gene, plays a key role in infection. Neutralizing antibodies to gB have no effect on the attachment of virions to cells but prevent the virion to penetrate cells. gB also promotes

transmission of infection from cell to cell, and fusion of infected cells (Navarro *et al.*, 1993). The gCII complex consists of at least two immunologically and biochemically distinct glycoproteins which are disulfide linked to each other (Kari *et al.*, 1990). One group of gCII glycoproteins may be encoded by a gene in the US6 gene family. This gene family is not essential for viral replication in culture (Kollert-Jons et al., 1991). The gene encoding the second glycoprotein has been identified as UL100 (Kari *et al.*, 1994). UL100 encodes for a 45-kD envelope protein. The exact role of this product is not known. The gCII complex is the major heparin binding component of the virion envelope, which is responsible for the initial interaction of the virus with the cell surface (Compton *et al.*, 1993).

One component of the gCIII complex is the glycoprotein gH, the product of gene UL75. The product of UL115, the CMV gL glycoprotein, forms a stable complex with gH and facilitates the transport of gH to the cell surface (Kaye ey al., 1992). Anti-gH antibodies can prevent cell to cell transmission of the virus (Rasmussen et al., 1991). These three glycoprotein complexes though are not essential for viral replication but they seem to play a very important role in the transmission, penetration and fusion of the virus with infected cells.

1.2.1 Infected cells and cells involved in latency.

CMV can establish both acute and chronic infections. After clearance of the infectious virus, the viral genome persists for life in the infected host, giving a possibility to reactivation of viral replication. During an active infection resulting in active viral production the salivary glands are an important place for growth and dissemination of CMV. During disseminated CMV infection viral antigens have been found in various organs. In lung tissue, epithelial cells and fibroblasts were the predominantly infected cells, while in gastrointestinal tissue mostly fibroblasts, endothelial, epithelial, and smooth muscle cells were infected. All these cell types might have the capacity to harbour CMV during latency and may be the source of recurrent infection.

While great progress has been made in the understanding of the mechanisms of CMV replication control and pathogenesis, our understanding of the mechanisms of establishment and maintenance of CMV latency and reactivation has remained incomplete and there is still controversy regarding the site of latency. Since CMV is transmitted with blood and organs from CMV-seropositive individuals, much *in vitro* research has focused on latency in blood cells. The general assumption concerning latency is that in latently infected cells viral DNA can be detected but no viral protein. As concerns CMV and cells of the immune system, *in vivo*, CMV interacts with endothelial cells monocytes, macrophages, polymorphonuclear leukocytes (PMNL) and bone marrow progenitor cells (Michelson, 1997). In 1991, Taylor Wiedeman *et al.*, for the first time detected the latent CMV genome in peripheral blood $CD14^+$ monocytes of healthy seropositive carriers but not in the T cell population of these subjects nor in the polymorphonuclear cells (Taylor-Weideman *et al.*, 1993). Monocytes positive for CMV DNA did not produce any viral RNA, but after *in vitro* stimulation and differentiation the monocytes-derived macrophages expressed immediate-early RNA transcripts. However, no transcripts encoding for other viral proteins could be detected, indicating that this does not lead to virus production (Taylor-Weideman *et al.*, 1994). Later studies showed, however, that monocytic cell precursors at specific developmental stages could be efficiently and productively infected *in vitro* with clinical isolates of CMV suggesting that *in vivo* monocytes may acquire CMV during their development in bone marrow (Movassagh *et al.*, 1996). Later viral DNA was found in $CD13^+$ and $CD34^+$ progenitor cells of monocytes/granulocytes in the blood and bone marrow which may be the site of latency rather than peripheral monocytes (Gerna *et al.*, 1992; Kondo *et al.*, 1996; Mendelson *et al.*, 1996; Torok-Storb *et al.*, 1992).

As early as 1984, Myerson *et al.* (1984) proposed that endothelial cells were the common denominator for viral dissemination; CMV antigens and nucleic acids (DNA and mRNA) have been detected in endothelial cells of numerous organs. Endothelial cells of blood vessel have been also discussed as a possible site of latency (Grefte *et al.*, 1994; Hackstein *et al.*, 1996).

In both normal and immunosuppressed individuals, numerous investigators have reported the detection of CMV in peripheral blood monocytes and PMNL using a variety of techniques including polymerase chain reaction (PCR); (Gerna *et al.*, 1992; von Laer *et al.*, 1995), *in situ* hybridization (Dankner *et al.*, 1990; Hendrix *et al.*, 1997; Link *et al.*, 1993; Turtinen *et al.*, 1987), *in situ* PCR (Yonemitsu *et al.*, 1996), virus isolation (Carney and Hirsch, 1981; Jordan, 1983) and detection of CMV antigens (Grefte *et al.*, 1992a,b; The *et al.*, 1990). CMV antigen-positive macrophages have been observed in the lung and spleen of normal seropositive subjects (Toorkey and Carrigan, 1989) and in the placenta of mothers prenatally infected with CMV (Sinzger *et al.*, 1996). CMV mRNA transcripts can be detected *in vivo* in bone marrow aspirates of some, but not all, healthy seropositive donors (Kondo *et al.*, 1996; Torok-Storb *et al.*, 1992).

1.2.2 Epidemiology.

Natural transmission of CMV occurs by direct or indirect person-to-person contact. CMV is very labile to heat and dryness, hence close or even intimate contact is believed to be required for its horizontal spread (Lang and Kummer, 1975). Sources of virus include oropharyngeal secretions, urine, cervical and vaginal excretions, semen, breast milk, tears, feces, and blood (Lang and Kummer, 1975; Stagno *et al.*, 1980). Human CMV infection is endemic and is present throughout the year rather than being seasonal (Gold and Nankervis, 1982).

1.2.3 Pathogenesis and pathology.

1.2.3.1 Normal Hosts.

The virus has a 4 to 8 week incubation period in normal older children and adults after viral exposure. In most cases, CMV infection in normal hosts leads to a clinically mild infection. Uncommonly, CMV infection in normal hosts results in the development of a mononucleosis syndrome clinically indistinguishable from the mononucleosis syndrome associated with EBV infection and may account for

approximately 8% of cases of infectious mononucleosis syndromes (Horwitz *et al.*, 1979; Klemola *et al.*, 1970). Persistent fever, myalgia, and nonspecific constitutional symptoms are common, as is cervical adenopathy. Infrequent complications of CMV mononucleosis include pneumonia, hepatitis, CNS involvement, aspetic meningitis, and a variety of immunologic abnormalities suggestive of autoantibody production (Klemola *et al.*, 1967, 1970; Phillips *et al.*, 1977). Laboratory findings include atypical lymphocytosis, and elevated hepatic transaminases (Klemola *et al.*, 1967, 1970; Phillips *et al.*, 1977).

1.2.3.2 Congenial and perinatal infections.

CMV infections in the foetus and the newborn may be severe. About 1% of live births annually in the USA have congenital CMV infections (Demmler, 1991; Yow *et al.*, 1988). About 10% of these suffer cytomegalic inclusion disease and a high percentage of babies with this disease exhibit developmental defects and mental retardation (Fowler *et al.*, 1992; Stagno *et al.*, 1986): Human CMV can be transmitted *in utero* with both primary and reactivated maternal infections (Stango *et al.*, 1986 and 1980; Yow *et al.*, 1988). Generalized cytomegalic inclusion disease (CID) results most often from primary maternal infections. Early in the course of infection, CMV is disseminated widely in various organs of the body. The virus may infect the placenta during infection and in some cases penetrate the placenta to infect the foetus.

1.2.3.3 Immunocompromised Hosts.

In man, CMV is an important pathogen in the immunologically immature and in the immunocompromised host (Meyers *et al.*, 1986). Serious disease is seen after intrauterine infection, in transplant patients and in individuals suffering from acquired immunodeficiency syndrome (AIDS) (Rubin, 1990). Manifestations are protean in that they can vary from asymptomatic excretion to manifestations in organs or organ systems such as in CMV-mononucleosis, CMV-retinitis,

interstitial pneumonia and disease of the gastrointestinal tract, and to general infection associated with multiple symptoms, as seen during congenital CID.

1.3 Role of CMV infection in the process of organ allograft rejection.

It was suggested that CMV infections might trigger rejection of transplant organs (Lautenschlager *et al.*, 1992). CMV infections are major infectious complications after organ transplantation, and a variety of clinical manifestations such as fever, leukopenia, thrombocytopenia, encephalitis, retinitis, pneumonia, hepatitis and glomerulopathy have been described as being associated with CMV infection (Rubin, 1990). In general, CMV is considered to be a significant risk factor in transplantation. An association between either acute or chronic rejection and CMV infection has been reported in several clinical series of renal (Poteil-Noble *et al.*, 1993), liver (O'Grandy *et al.*, 1988) and heart (Grattan *et al.*, 1989) transplantations.

It should be emphasized that with the use of improved immunosuppressive protocols and effective anti-rejection therapies, the results of organ transplantation have progressively improved, yet the problems of CMV infection in organ transplant patients have not been resolved.

1.3.1 CMV in renal transplantation.

CMV infection is closely linked to acute rejection. Rejection favours CMV infection due to the increased immunosuppressive treatment that usually accompanies signs of transplant rejection. Acute rejection is observed from the second week and chiefly during the first 2 months after transplantation. The perception of swollen kidneys is infrequent, as is fever. In practical terms and in most cases, infection is associated with changes in renal functions: reduced diuresis, increased creatininaemia, reduced natriuresis. In renal transplantation CMV has been suggested to cause graft glomerulopathy associated with viremia

(Richardson *et al.*, 1981) and to trigger the immune mechanisms of acute rejection in kidney transplant patients (Pouteil-Noble *et al.*, 1993).

CMV infectivity has been studied in various cellular components of the kidney. Human glomerular, mesangial, tubular and endothelial cells were isolated and infected with human CMV strain AD 169 (Ustinov *et al.*, 1991). All types of human renal cellular components could be easily infected with CMV (Ustinov *et al.*, 1991) as had been shown with *in vitro* studies (Heieren *et al.*, 1988a,b; Ho *et al.*, 1984) which demonstrated that several types of tissue cells such as mesangial cells and endothelial cells, can be readily infected by CMV.

CMV infection contributes not only to the onset of acute rejection but also to chronic rejection episodes. Chronic rejection of renal allografts is characterized by a progressive worsening of the renal function, typically beginning in the first 3 to 6 months following transplantation, but sometimes much later. In chronic rejection the following clinical picture may appear: high blood pressure, a proteinuria, a gradual increase in creatininaemia evidencing a progressive reduction of glomerular filtration rate. To ascertain whether CMV infection has any clinical impact on the appearance of acute or chronic rejection in renal transplantation, 290 consecutive kidney allograft recipients transplanted during 2 years were analyzed (Lautenschlager *et al.*, 1992). The follow up time was 3 years. Of these, 42 developed a virologically confirmed (culture and/or pp65 AA (section 1.4)) infection with active CMV infection (80%) appearing during the first 3 months (80±22days). However, in the CMV infected group, 45% of the patients underwent signs of acute rejection during the first year, but only one graft was rejected. In the CMV-infected patients with chronic rejection 4/42 grafts were rejected. The differences were not considered significant. However, in another analysis of a large clinical material in Helsinki Center, consisting of 1,177 adult renal transplant patients with an 8 year follow up, CMV infection (culture and/or pp65 AA (section 1.4)) had a significant effect on long-term grafts survival and the development of chronic rejection (Isoniemi et al., 1997).

1.3.2 CMV in heart transplantation.

Though clinical studies have shown that acute rejections are more frequent in transplant patients with CMV infection than in those without (Grattan *et al.*, 1989), the most important is the role of CMV in chronic rejection also called cardiac allograft vasculopathy. CMV infection is most closely associated with cardiac allograft vasculopathy which is characterized by arteriosclerotic changes (Everett *et al.*, 1992). In a series of clinical studies, including 80 heart transplant patients, CMV was shown to be linked with histopathological changes of the vascular wall in human endomyocardial biopsies (Koskinen *et al.*, 1993a). CMV was associated with accelerated arterial thickening of the small intramyocardial arteries and subendothelial inflammation of the vascular wall (Koskinen *et al.*, 1993b).

1.3.3 CMV in liver transplantation.

The liver is a preferred site for CMV replication, and liver grafts have been found to be the most commonly affected organ in primary CMV infections (Stratta *et al.*, 1989). In addition to a variety of other CMV-associated clinical manifestations, CMV hepatitis is a common finding in liver transplant patients (Paya *et al.*, 1989). Thus, the graft itself is often affected both directly by the virus and also by the immune response against the virus, and this may cause diagnostic problems. The most common differential diagnostic question in liver transplantation is whether loss of the organ is due to rejection or to viral infection (Lautenschlager *et al.*, 1990). Liver allografts were monitored by frequent fine-needle aspiration biopsies and transplant aspiration cytology to demonstrate the inflammation associated either with acute rejection or viral infection. Acute rejections appeared shortly (7±3 days) after transplantation with a high peak of inflammation and intense lymphoid activation in the graft, which subsided within a few days with antirejection therapy. CMV infection, demonstrated by the pp65 AA (section 1.4), developed later (33±11 days) after transplantation with mild

lymphoid activation recorded not only in the graft but also in the peripheral blood of the patient. The inflammation subsided slowly during antiviral therapy with ganciclovir. CMV infection seemed not to be linked to early acute rejection episodes. The importance of concomitant CMV infection and late acute rejections has been reported by others (Cakalogu *et al.*, 1995).

Some years ago, an association between CMV infection and chronic liver allograft rejection was reported (O'Grady *et al.*, 1988). Furthermore, persistent CMV DNA was found in the hepatocytes of liver graft with chronic rejection (Arnold *et al.*, 1992). Recently, *in situ* DNA hybridization demonstrated that the CMV genome persists not only in hepatocytes but also in the bile ducts and vascular endothelial cells (Lautenschlager *et al.*, 1997a). As persistent CMV DNA was found just in those structures that are targets for chronic rejection, these findings support the suggestion of involvement of CMV in the process. CMV may cause direct damage to the cells, or may activate immune responses against infected cells, or the mild immune response against the virus may activate various cytokines, which leads to increased expression of human lymphocyte antigens (HLA) thus triggering the mechanisms of alloresponse.

1.3.4 CMV in bone marrow transplantation.

CMV infection is one of the major threats to the recipient of an allogeneic stem cell graft (Boeckh *et al.*, 1996; Meyers *et al.*, 1986;). The incidence of CMV infection increases with intensity and duration of immunosuppression and approaches 70% in allogeneic bone marrow transplant (BMT) recipients who are either CMV-seropositive and/or receiving a transplant from a CMV-seropositive donor (Meyers *et al.*, 1986). CMV disease is still associated with significant morbidity in these high-risk patients and, in spite of combined treatment with ganciclovir and high-dose immunoglobulin, also with a high fatality rate in recipients of an allogeneic stem cell transplant (Ljungman *et al.*, 1992, 1994). CMV-associated disease consisting of fever, malaise, atypical lymphocytosis, leukopenia, myalgia and arthralgia is frequently observed in recipients of BMT and

often precede CMV enteritis or CMV-induced interstitial pneumonitis. The most threatening CMV-associated complication following bone marrow transplantation is CMV-induced interstitial pneumonia, which develops 7-10 weeks after transplantation. The mortality of CMV-induced interstitial pneumonia after bone marrow transplantation exceeds 70% (Meyers et al., 1986). Strategies have been evaluated to prevent CMV disease with chemotherapy (ganciclovir and/or foscarnet), either administered prophylactically or early (preemptive therapy). Preemptive antiviral therapy based on sensitive screening tests (Boeckh *et al.*, 1996) as well as antiviral chemotherapy (Goodrich *et al.*, 1993) have been shown to significantly reduce CMV disease in high risk patients.

1.3.5 CMV in experimental models of transplantation.

Animal models have been used to investigate the role of CMV infection in renal transplantation. In experimental models of mouse and rat for CMV infection in allograft recipients it has been demonstrated that latent CMV is transferable through kidney allografts (Bruning *et al.*, 1989; Hamilton and Seaworth, 1985). It has been suggested that the infection is brought to the graft by inflammatory cells and that the virus, carried by a small number of cells in the graft, may be reactivated by allogenic stimulation and immunosuppression (Bruning *et al.*, 1989).

Experimental studies have been mainly focused on the effect of CMV on the ongoing process of chronic rejection of the kidney allograft in a rat animal model that resembles the clinical situation as closely as possible (Lautenschlager *et al.*, 1997b). This included triple drug immunosuppression by steroids, azathioprine and cyclosporin similar to that used with patients in most transplant centres. The use of cyclosporin alone is considered unadvisable because it causes long-term changes in the graft, which cannot always be distinguished from those of chronic rejection (Mihatsch *et al.*, 1995). A rat model of chronic renal allograft rejection was developed (Soots *et al.*, 1997) in which, by using a certain rat strain combination and mild triple drug therapy, the grafts showed chronic allograft damage within

40-60 days, fulfilling the histological criteria of chronic rejection according to the Banff criteria (Solez *et al.*, 1993). The chronic changes of the graft were evaluated by the chronic allograft damage index, which is derived from 6 histopathological changes characteristic for chronic renal allograft rejection. These are interstitial inflammation and fibrosis, glomerular sclerosis and mesangial matrix increase, vascular intimal thickening and tubular necrosis (Isoniemi *et al.*, 1994). The model of rat CMV (RCMV) infection (Bruggeman *et al.*, 1983) was then applied to this experimental model of renal transplantation. RCMV infection increased the inflammation monitored by frequent fine-needle aspiration biopsy cytology (von Willebrand and Lautenschlager, 1996), increased the macrophage response, generated fibrosis and narrowed the arteries and arterioles significantly earlier in infected transplant rats than these occurred in uninfected transplant rats. Histologically, chronic rejection was diagnosed as early as 20 days after transplantation in CMV-infected grafts (Lautenschlager *et al.*, 1997b). One characteristic finding associated with CMV infection, and not seen in uninfected grafts, was the early (5-7 days after transplantation) medical necrosis of the large arteries (arteria renalis). This study also demonstrated the gradual development of RCMV infection in the graft, the virus first seen in the tubular cells and thereafter in the arteries, until at day 20 the graft was rejected (Lautenschlager *et al.*, 1997b).

RCMV infection (von Willebrand and Lautenschlager, 1996) was also tested in the experimental transplantation of rat aorta allografts. Without immunosuppression these slowly developed arteriosclerotic alterations such as persistent perivascular inflammation, smooth muscle cell proliferation, and intimal thickening, which are similar to that of the heart transplant model (Mennander *et al.*, 1991). In this model it was demonstrated that RCMV accelerated the chronic rejection of vascular allografts, induced a prominent inflammatory response in the rat aorta allograft, doubled the proliferation of smooth muscle cells and induced intimal thickening (Lemström *et al.*, 1993).

When the rats received basic triple drug immunosuppression treatment the enhancement of the inflammation was reduced, as well as the arteriosclerotic

changes (Lemström *et al.*, 1994). This experiment also demonstrated the important role of the immune response in the phenomena associated with CMV.

To study the effect of CMV in a standardized experimental model of hepatic transplantation, RCMV infection was used to study acute rejection of liver allografts in the rat (Martelius *et al.*, 1997b). When the animals were infected with RCMV, the portal inflammation associated with acute rejection was significantly increased and also more severe bile duct damage was recorded in the graft (Martelius *et al.*, 1997a).

1.4 Laboratory diagnosis of CMV infection.

The best means of diagnosing CMV infection is virus isolation. The virus can be recovered most readily from throat washings and urine. Other body fluids, as well as biopsy materials, sometimes yield CMV. In culture, 1-2 weeks are usually needed for the appearance of cytological changes, and these consist of small foci of swollen, translucent cells with large intranuclear inclusions (Griffiths, 1984). Immunofluorescence is frequently used to demonstrate CMV antigen directly from clinical samples and often there is an advantage in combining virus isolation and immunofluorescence. In particular, immunofluorescence examination of inoculated culture will markedly decrease the usual time required for demonstration of cytopathic effects.

Several serological methods (complement fixation test, radioimmunoassay, immunofluorescence and ELISA techniques) may be used to detect CMV-specific IgM or IgG antibody responses. Neutralization tests for antibody detection are less reliable because the preparations of CMV employed must be free of infected cells, which is difficult because standard virus suspensions contain mainly cell-associated virus.

The increase in the number of patients with AIDS or organ transplants has resulted in an increase in opportunities to consider the diagnosis of CMV infection, and the possibility of effective therapy if infection is diagnosed sufficiently early has heightened the need for accurate and rapid laboratory methods for diagnosis of

viral infection. In addition, the well-established epidemiology of CMV transmission from blood transfusions (Alder, 1983) and donor organs (Chou, 1986) has created a continuing need for serologic methods suitable for screening donors and recipients in efforts to avoid adverse clinical outcomes in immunodeficient recipients.

Rapid and accurate diagnosis of active CMV infection in transplant recipients and in patients with autoimmune diseases is of great importance in avoiding over-treatment with immunosuppressive drugs and in guiding antiviral therapy. For these reasons rapid and sensitive methods of detecting the virus have been developed. Apart from virus isolation and serological methods, the diagnosis of an active CMV infection can be achieved by utilizing the presence of CMV structural antigens in peripheral blood leukocytes (van der Bij 1988b). In peripheral blood leukocytes of heart transplant patients with CMV infection a CMV-specific antigen was detected by monoclonal antibodies to CMV IE antigen. This antigen has been identified as a 65 kD viral matrix phosphoprotein (pp65) (van der Bij 1988b). Monoclonal antibodies react with the pp65 antigen expressed mainly in blood leukocytes (van der Bij 1988a,b). This method seems to have been a breakthrough in the rapid diagnosis of systemic CMV infections in immunocompromised patients. The method, known as the CMV AA is extremely useful for detecting active CMV infection in transplant patients. The assay consists of the following four steps, 1) isolation of blood leukocytes; 2) preparation of microscopic slides; 3) immunoperoxidase staining with the use of murine monoclonal antibodies to CMV immediate early antigen (pp65); 4) evaluation and semiquantitative scoring (van der Bij *et al.*, 1988a, b). On average, the test provides diagnostic information about 1 week earlier than other methods. The major drawback of the AA is the need for immediate processing of blood samples to achieve optimal sensitivity and the consideration of certain technical aspects to avoid pitfalls (Boeckh *et al.*, 1992).

Among the different techniques used to confirm an active CMV infection, the detection of the CMV pp65 has the advantage of quantifying the viral load according to the number of antigen containing cells, which correlates well with

manifestations of CMV disease (Gerna *et al.*, 1992). However, the quantification of antigen-positive cells by the AA is subjective and demands very careful and time-consuming microscopic examination (Gerna *et al.*, 1992). With the advent of effective antiviral chemotherapy for human CMV infection, more rapid and sensitive techniques are required to identify CMV infection during the acute stage of the illness and also to monitor antiviral therapy (Boeckh *et al.*, 1996). DNA amplification by the polymerase chain reaction (PCR) has proved to be a reliable method to detect CMV genome in clinical materials (Einsele *et al.*, 1991a,b). These assays have helped guide new antiviral strategies and improve patient management (Einsele *et al.*, 1991a,b). More recently quantitative PCR assays have become available (Gerna *et al.*, 1995).

Serological testing is also used to confirm exposure to CMV but this requires both acute and convalescent sera, demonstrating a fourfold rise in titre. This technique is less reliable since immunosuppressed patients may not have a change in antibody titre even in the presence of an active CMV infection (Drew *et al.*, 1985).

The use of flow cytometry in the detection of CMV infections has grown to significant proportions (Elmendorf *et al.*, 1988; Jiwa et al., 1994; McSharry, 1994; Ploem-Zaaijaer *et al.*, 1994).

1.5 Immunity to CMV.

It has been established from experiments with murine CMV (MCMV) that under certain experimental conditions natural defense mechanisms such as genetic resistance and natural killer cells (NK cells) can considerably contribute to resistance against lethal infection with a high dose of virus (Bukowski *et al.*, 1984; Scalzo *et al.*, 1992). The absence of a specific immune response despite the presence of NK cells, eventually results in a fatal outcome (Welsh *et al.*, 1991). Therefore, attention has concentrated in recent years on situations in which the critical parameter is not the initial dose of infectious virus, but the capacity to limit

virus growth and spread in an antigen-specific manner. This has focused on antibodies and on the role of T lymphocytes in the control of CMV infection.

1.5.1 Humoral immunity.

The humoral immunity system affords protective immunity by the generation of neutralizing antibodies, which can interact either with free virus particles, or with viral glycoproteins expressed on the surface of infected cells. Antibodies directed against the envelope glycoprotiens frequently neutralize the virus *in vitro* (Middeldrop *et al.*, 1986; Navarro *et al.*, 1993). For CMV, the majority of neutralizing antibodies described to date are specific for the CMV glycoproteins gB (UL55) (Utz *et al.*, 1989) or gH (UL75) (Hamilton *et al.*, 1997), which are expressed both on the viral envelope, and the infected cell surface. In addition to neutralizing free virus particles, some antibodies have been shown to prevent cell-to-cell spread of the virus (Navarro *et al.*, 1993). Antibody might also play a role in killing CMV-infected cells, by either antibody plus complement-mediated cell lysis (Middeldorp *et al.*, 1986), or antibody-dependent cellular cytotoxicity (Kirmani *et al.*, 1981), both of which have been demonstrated to occur *in vitro*.

A potential evasion mechanism to evade humoral immunity is the induction of a receptor for the Fc portion of IgG by CMV (MacCormac and Grundy, 1996; Sakuma *et al.*, 1977). The Fc receptor has been demonstrated on the surface of infected endothelial cells and fibroblasts (MacCormac and Grundy, 1996). It is not known whether the CMV Fc receptor is also present on the viral envelope, where it could function to prevent the binding of neutralizing antibodies.

1.5.2 Cell-mediated immunity.

While infections by CMV are often unnoticed in immunocompetent hosts, where it causes only mild or subclinical disease, in immunocompromised patients it may cause severe clinical symptoms (Meyers *et al.*, 1986). Following a primary infection the virus remains in a latent state and is subsequently reactivated in

immunosuppressed patients undergoing transplantation or in conditions such as cancer or AIDS (Rubin, 1990). However, the mechanisms of tissue injury caused by CMV infection in renal transplant recipients are not well understood. Host immune responses to CMV infection involve different compartments of the immune system. Studies in immunosuppressed humans and animals have revealed that $CD4^+$ and $CD8^+$ T cells are of crucial importance in the maintenance of immunity to CMV as well as for the eradication of an ongoing infection (Borysiewicz *et al.*, 1988; Jonjic *et al.*, 1989 and 1990). In human and animal experiments, the adoptive transfer of syngeneic, polyclonal $CD8^+$ T cells to immunosuppressed individuals has been shown to provide protection from CMV disease (Quinnan *et al.*, 1982; Reusser *et al.*, 1991). Cytotoxic $CD8^+$ T cells have been described as being important effectors of the anti-CMV response and are mostly directed against matrix proteins such as pp65 (Doherty *et al.*, 1992; Reusser *et al.*, 1991, Riddell and Greenberg, 1994, Wills *et al.*, 1996). Adoptive immunotherapy with CMV-specific $CD8^+$ T cell clones from allogeneic donors has been shown to prevent the development of CMV disease (Reddehase *et al.*, 1985, 1987). In addition, allogeneic transfer of CMV-specific $CD8^+$ cell clones has been found to reconstitute cellular immunity against CMV *in vivo* (Riddell *et al.*, 1992). It has also been shown that infusion of increasing doses of T-cell clones results in an increased CMV-specific cytotoxic T lymphocyte response in recipients (Walter *et al.*, 1995).

1.5.3 CMV persistence: escape from cell-mediated immunosurveillance.

At the effector level, the immune system must either deal with free virus particles, rendering them non-infectious, or virus-infected cells, which must be killed before the virus has replicated. Neutrophils and macrophages can take up and destroy many free infectious agents, however CMV can survive in both of these cell types, and this part of the innate immune system is thus not effective in dealing with CMV. The potentially protective effector mechanisms of defense

against CMV and the mechanisms CMV can use to evade them are discussed below.

1.5.3.1 Human CMV inhibits the expression of MHC class I molecules.

Based on the observation that 1) MHC class I is reduced on the surface of CMV infected cells, 2) there is a perinuclear retention of MHC class I, 3) the association of β_2-microglobulin (β_2m, 12kD) with class I heavy chain (HC) is not affected by CMV infection, and 4) that there are large decreases in steady-state levels of class I HC without a reduction in class I HC mRNA have fueled investigations into the mechanisms mediating these phenomena (Barnes and Grundy, 1992; Beersma *et al.*, 1993; Yamashita *et al.*, 1993). Metabolic labeling analyses show that the reduction in free HC is the result of accelerated degradation and this phenotype correlates with CMV IE or E gene products (Beersma *et al.*, 1993; Yamashita *et al.*, 1993).

The principle structure of CMV-genome is the unique nucleotide sequence, the long UL and the short US. To identify genes responsible for the class I degradation, Jones *et al.*, (1995) screened a panel of CMV deletion mutants, each unable to express one of 18 nonessential open reading frames. The products of two genes, US2 and US11, were shown to independently decrease MHC class I expression by mediating the cytosolic degradation of HC by the proteosome. US2 and US11 have a high degree of homology with another CMV glycoprotein, US3 (Ahn *et al.*, 1996) and it has been speculated that the US3 protein functions in an analogous fashion. However, the US3 protein does not enhance degradation of MHC class I molecules, rather it retains β_2m-associated HC in the endoplasmic reticulum (ER) (Ahn *et al.*, 1996; Jones *et al.*, 1996; Tenney and Colberg-Poley, 1991). In addition to inhibiting MHC class I expression, the CMV early US6 glycoprotein disrupted the transport of proteasome-generated peptides into the ER (Ahn *et al.*, 1997; Hengel *et al.*, 1997). Peptide translocation was restored in a recombinant CMV lacking US6 (Ahn *et al.*, 1997).

Based on the analyses of the functions of the US2, US3, US6 and US11 gene a model of CMV inhibition of MHC class I expression has been developed. During immediate early times postinfection the US3 gene product retains β_2m-associated HC in the ER. At early times postinfection US2 and US11 proteins target predominantly free HC for proteolytic degradation and US6 disrupt the transport of polyosome generated peptides to ER. Furthermore, there is cooperation between the US3, US2 and US11 gene products at early-late times postinfection since the US2 and US11 proteins shuttle US3-retained class I HC to the cytosol for degradation (Wiertz *et al.*, 1996a,b).

1.5.3.2 CMV escapes $CD8^+$ cytotoxic T lymphocyte lysis.

Cell-mediated immunity is critical to the control of CMV infection and in individuals with depleted lymphocyte number and function (e.g. AIDS patients and transplant recipients), (Doherty *et al.*, 1992; Li *et al.*, 1994). $CD8^+$ T lymphocytes play a critical role in the antiviral effector arm of the adaptive immune system, i.e. CMV-specific, MHC class I restricted $CD8^+$ cytotoxic T lymphocytes (CTL) lyse CMV-infected cells (Borysiewicz *et al.*, 1988; Doherty *et al.*, 1992; Reusser *et al.*, 1991; Riddell *et al.*, 1992). The importance of CMV-specific CTL is established by their ability to limit the severity of CMV infection in immunocompromised hosts (Quinnan *et al.*, 1982; Riddell *et al.*, 1992, 1994).

$CD8^+$ CTLs target peptides derived from CMV proteins presented in the context of the MHC class I HC molecule (Kootstra *et al.*, 1997). Peptides are generated in a proteasome complex, a multicatalytic protease that mediates the adenosine triphosphate (ATP)-dependent degradation of ubiquitin-conjugated protein (Heemels and Ploegh, 1995). Cytosolic proteins are degraded by the proteasome and peptides are translocated into ER to be loaded onto MHC class I heterodimers by the transporters associated with antigen processing (TAP) (Heemels and Ploegh, 1995). After their synthesis in the ER, MHC class I molecules are loaded with peptides of 8-11 amino acids, forming a stable trimolecular complex (Kootstra *et al.*, 1997).

Inhibition of MHC class I molecule expression is a common theme in viral immunoevasive mechanisms. Both DNA viruses (adenovirus, herpesvirus, papovavirus, and poxvirus) and RNA viruses (coronavirus, rhabdovirus, paramyxovirus, and retrovirus) inhibit MHC class I expression (reviewed in Rinaldo, 1994). The widespread use of this mechanism by divergent viruses supports the proposal that decreasing MHC class I expression provides a selective survival advantage to the microorganism, allowing the evasion of MHC class I restricted $CD8^+$ CTL immunosurveillance.

1.5.3.3 CMV evades NK cell lysis.

NK cells can recognize and kill virus-infected cells, and CMV-infected cells have been shown to be killed by NK cells *in vitro* (Borysiewicz *et al.*, 1986; Starr and Garrabrant, 1981). The killing of CMV-infected target cells *in vitro* by NK cells differs from that of other target cells, in that a prolonged assay period of approximately 18 hours (rather than the usual 4 hours) is needed before killing can be demonstrated (Starr and Garrabrant, 1981). It is thought that some form of activation of the NK cells is necessary before lysis can occur and there is some evidence for a protective role of NK cells against CMV disease in transplant recipients *in vivo* (Quinnan *et al.*, 1982).

NK cells lyse target cells which express little or no MHC class I molecules, i.e. 'missing self' (Scalz *et al.*, 1992; Weinberg *et al.*, 1996). NK cells, induced immediately following CMV infection lyse CMV-infected cells during acute infection (Bancroft *et al.*, 1981; Scalzo *et al.*, 1992; Weinberg *et al.*, 1996) and are an essential component of the acute response to viral infection (Bancroft *et al.*, 1981; Bukowski *et al.*, 1984).

The identification of an CMV encoded glycoprotein (from gene UL18) homologous to MHC class I was a breakthrough discovery in understanding how CMV-infected cells escape NK cell lysis (Beck and Barrel, 1988). The UL18 protein binds endogenous β_2m and carries it to the surface of the infected cell (Browne *et al* 1990; Grundy *et al.*, 1987a). Where the UL18/β_2m heterodimers are loaded with peptides, the profile of which is similar to cytoplasmic proteins typically found in the context of MHC class I heterodimers (Fahnestock *et al.*, 1995).

Given that MHC class I negative CMV-infected cells are targets of NK cells it was hypothesized that the UL18/β_2m/peptide complex protected CMV-infected cells from NK cell lysis (Fahnestock *et al.*, 1995). Experiments have been performed to directly assess the role of UL18 in inhibiting NK cell lysis have shown that MHC class I negative B cell line transfected with UL18 confers resistance to lysis by NK cells (Reyburn *et al.*, 1997).

CMV and MCMV encode MHC class I homologues which protect infected cells from NK cell lysis. The effect of the UL18 protein *in vivo* was characterized by utilizing deletion mutants of MCMV. m144 is the MCMV MHC class I homology (Farrell *et al.*, 1997). Replication of these mutants during acute infections is severely restricted by NK cells in visceral organs of BALB/c mice 2-6 days postinfection, with titres 400-fold lower than wild type virus (Farrell *et al.*, 1997).

The data suggest that m144-induced inhibition of NK cell lysis is critical to maintenance of MCMV in visceral organs during the acute phase of infection when innate immunity constitutes the major antiviral effector mechanism (Farrell *et al.*, 1997). CMV infection confers resistance to NK-cell-mediated cytolysis *in vitro* and *in vivo* (Farrell *et al.*, 1997; Reyburn *et al.*, 1997). In addition to the UL18 and m144 genes, it has been proposed that the MCMV gp34/MHC class I HC complex also inhibits NK cell lysis (Kleijnen *et al.*, 1997).

1.5.3.4 CMV escapes $CD4^+$ T lymphocyte immunosurveillance.

$CD4^+$ T helper cell subsets play important roles in both humoral and cell-mediated immunity. Murine $CD4^+$ T cells participate in the clearance of CMV from salivary glands (Lucin *et al.*, 1992), while human $CD4^+$ T cells have been reported to play a role in the defence against intracellular pathogens (Pieters, 1997). However, the participation of $CD4^+$ T cells in responses to CMV infection, through the secretion of cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) and mechanisms such as cytotoxicity and helping B and $CD8^+$ cells are still not well established (Fish *et al.*, 1996).

$CD4^+$ T cells recognize antigens presented in the context of MHC class II molecules (Pieters, 1997). Professional antigen-presenting cells such as dendritic cells, macrophages, B cells, and thymic epithelial cells constitutively express MHC class II molecules (Glimcher and Kara, 1992; Boss, 1997). MHC class II molecules are highly polymorphic heterodimers consisting of α and β-chain (Pieters, 1997). For inducible antigen-presenting cells, such as activated T cells, endothelial cells, and epithelial cells, IFN-γ is the most potent inducer of MHC class II molecules (Boss, 1997; Glimcher and Kara, 1992).

$CD4^+$ T lymphocytes are a requisite component of anti-CMV immunity (Jonjic *et al.*, 1989). The primary role of $CD4^+$ T cells in CMV infection is an afferent one, augmenting $CD8^+$ T lymphocyte and B lymphocyte responses to CMV infection. A more direct role of $CD4^+$ T cells in mediating viral clearance may be due to MHC class II restricted cytolysis or direct antiviral effects of the T helper (Th) 1-type cytokines released by CMV-activated $CD4^+$ T cells (Davignon *et al.*, 1996; Lucin *et al.*, 1992; Muller *et al.*, 1992; Rinaldo, 1994). CMV infection inhibits MHC class II expression in arterial and venous endothelial cells *in vitro* and *in vivo* (Knight *et al.*, 1997; Sedmak *et al.*, 1994). Infected endothelial cells treated with IFN-γ lack class II antigens on their surface and in their cytoplasm (Sedmak *et al.*, 1994).

Also, there is accumulating evidence that CMV disrupts MHC class II expression in monocytes, which constitutively express MHC class II molecules. CMV-infected monocytes lack cytoplasmic and surface expression of MHC class II molecules but the mechanism of this is presently unknown (Fish *et al.*, 1996). CMV-infected cells escape $CD4^+$ T cell immunosurveillance, which appear to be a conserved immunoevasive strategy that provides a survival advantage to the pathogen (Slater *et al.*, 1991).

1.5.3.5 Mechanism of immunosuppression by CMV.

Escape from immunosurveillance by T lymphocytes and lysis by NK cell are critical immunoevasive strategies for intracellular microorganisms. CMV has evolved diverse mechanisms for evading host cellular immunity: 1) Lysis by $CD8^+$ CTL is inhibited by multiple glycoproteins within the unique short component of the CMV genome that inhibits MHC class I expression, 2) By surface expression of the protein UL18 and m144; 3) $CD4^+$ T cell immunosurveillance is inhibited by disrupting IFN-γ inducible and constitutive MHC class II expression. The establishment of a latent or persistent infection requires subversion of host immunity. The finding that divergent viruses have coevolved analogous means of escaping cellular immunity emphasizes that there are key immune functions which must be disabled in achieving a persistent infection or reactivation of a latent virus.

When looking at the mechanisms of immune suppression which occur during acute infection by CMV, it is relevant to consider that what the virus does to modulate the immune system is a reflection of its way to survive. Like all herpesviruses, CMV becomes a permanent resident of its host following primary infection. This implies that the virus must not only escape immune responses aimed at its elimination, but that it must also have devised ways to control its own level of replication, and hence its expression, to evade detection by the immune system.

1.5.3.6 Possible mechanisms involved in CMV infection and graft/transplant rejection.

Several mechanisms have been suggested to explain the relationship between CMV infection and graft rejection. The two main possibilities are either that the virus acts as an adjuvant and triggers the rejection cascade in the allograft or that the rejection activates the latent virus. Whether one of the mechanisms, or even both of them, are involved has not yet been established. A great number of *in vivo* and *in vitro* studies support the hypothesis that CMV may trigger rejection. In particular, increased expression of MHC class I and II antigens on lymphocytes which have been noted during allograft rejection but also in association with CMV infection (Grundy *et al.*, 1988a; van Dorp *et al.*, 1989; van Es *et al.*, 1984; von Willebrand 1986).

In the early studies, the upregulation of MHC class I and II antigens, mediated by IFN–γ, was thought to be the key to understand the mechanisms (van Es *et al.*, 1984; von Willebrand 1986). Large amounts of IFN-γ are produced by activated lymphocytes during viral infections, but also during rejection episodes (Hall *et al.*, 1984). Class II induction has been demonstrated to be crucial for allograft rejection (Hall *et al.*, 1984), and an increase of foreign class II was simply thought to increase the immunogenicity of allograft and trigger rejection. It seems that CMV alone cannot induce class II expression of cultured human endothelial cells *in vitro* (Ustinov *et al.*, 1991; van Drop *et al.*, 1989), and inflammatory cells are needed to be present to induce this process (Waldman *et al.*, 1993). These inflammatory cells are always present in the allograft, especially if there is an ongoing alloresponse. Thus, the viral infection in the graft induces or increases the expression of MHC antigens on the cell surface and triggers the antiallograft response or enhances the already ongoing rejection. The fact that rejection often precedes the clinical evidence of CMV infection could be explained by the long incubation time of several weeks, as the virus usually is of donor origin and is received with the graft at the moment of transplantation (Grundy *et al.*, 1988b). It could be suggested that the very early phase of the infection, or even the latent

virus, may already induce MHC antigen expression, both class I and II, before the clinical symptoms and laboratory diagnosis of the infection. On the other hand, CMV has also been demonstrated to suppress MHC antigen expression in infected cells (Scholz *et al.*, 1992; Sedmak *et al.*, 1994; Yamashita *et al.*, 1993).

Another suggested mechanism is that allograft rejection causes the infection by activating the latent virus is also posssible. In experimental models of mouse and rat CMV infection in allograft recipients it has been demonstrated that latent CMV is transferable through kidney allograft (Hamilton and Seaworth 1985). It is suggested that the infection is brought to the graft by the inflammatory cells and that the virus carried by a small number of cells in the graft may be reactivated by allogenic stimulation and immunosuppression (Bruning *et al.*, 1989). The alloreactive lymphocytes then produce IFN-γ and cause the increase of MHC antigens, also class I antigens (Grundy *et al.*, 1988a). The class I molecules is especially important in the immune response against the virus to mediate cytotoxic lymphocytes or natural killer cells (Quinann *et al.*, 1984). The class I molecule, or the β_2m chain of it, has also been suggested to be the receptor for CMV (Grundy *et al.*, 1987b), and CMV has been demonstrated to bind to β_2m molecules (Grundy *et al.*, 1987a). The increase of the possible receptors on the cell surfaces in the graft could facilitate the viral binding and promote the infection. In man, the importance of inflammatory cells in CMV infection has been confirmed by a study demonstrating that the principal CMV-infected cells in the kidneys of renal transplant patients with primary CMV infections are infiltrating inflammatory cells (Gnann *et al.*, 1988).

Further information on the mechanisms by which CMV infection may contribute to graft rejection has been published. DNA sequence analyses have demonstrated that CMV encodes a molecule similar to the MHC class I antigen (Beck and Barrel 1988). This molecule has been suggested to bind β_2m and could be involved with virus-cell attachment. On the other hand, this molecule might interfere with the induction of cell-mediated MHC-restricted immunity and cytolytic T cell response against the infected cells (section 1.5.2.3). In addition, sequence homology and immunologic cross-reactivity of CMV with the HLA-DR

β-chain have been demonstrated (Fujinami *et al.*, 1988). The viral IE-antigen-induced antibodies have been found to recognize the DR antigens. This phenomenon could explain some mechanisms of autoimmunity associated with viral infections. The cross-reactivity would also fit in the allograft rejection mechanisms.

Thus, at the early phase of immune activation either due to alloresponse or to immune response against the virus, both of these factors can exacerbate the immune response. On te other hand, the inflammatory cells infiltrating the graft during the alloresponse may bring the latent virus to the graft, and the virus is activated by the cytokines produced during the early phase of alloresponse.

1.6 CMV and cytokines.

1.6.1 Cytokines.

Cytokines are peptide or glycopeptide mediators of relatively low molecular mass (rarely more than 8-25 kD) and often consisting of just a single chain. The cytokine family includes a large (and still growing) number of proteinaceous intracellular signaling molecules that mediate a variety of actions with and without the immune system. Because no brief definition can encompass all features of the members of this very diverse family, cytokines are best defined by a list of their characteristic properties (Table 2). Cytokines are produced by a variety of different cells and very often several unrelated cell types can produce the same cytokine. The nomenclature of cytokines reflects its evolution from many distinct sources (Vilcek and Le, 1994).

Th1 and Th2 cells are the major, functional subsets of T helper cells (Mosmann and Coffman, 1989; Romagnani, 1992); Th1 cells induce several cytotoxic and inflammatory reactions by the production of IL-2, IL-12, IFN-γ and TNF, and are responsible for cell-mediated inflammatory reactions, delayed-type hypersensitivity and tissue injury in infectious and autoimmune diseases. In contrast, activation of Th2 subsets of T cells results in the production of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, which stimulates predominantly humoral immune

responses mediated by antibodies. Th1 and Th2 subsets are of major importance in determining the class of immunoprotective function in infectious diseases. Thus, a bias either towards Th1-dominance or Th2-dominance can make a difference between effective elimination of the virus and exacerbation of the disease (Mosmann and Sad, 1996). Research on HIV infection (Clerici and Shearer., 1993), murine AIDS (Gazzinelli *et al.*, 1992), vaccinia virus (Actor *et al.*, 1994), herpes simplex virus (Jayaraman *et al.*, 1993) and influenza virus (Graham *et al.*, 1994) has shown that generally Th2 responses exacerbate the infection, while Th1 responses are protective.

1.6.2 Modulation of cytokine levels by CMV.

It is hard to assess the effect of CMV infection on cytokine production *in vivo*. Observations made on *in vitro* studies may appear clearer, but when extrapolated to *in vivo* animal models, the results are sometimes surprising and even contradictory (Haagmans *et al.*, 1994; Pavic *et al.*, 1993; Price *et al.*, 1996; Shanley *et al.*, 1994; Yerkovich *et al.*, 1997). Two aspects of CMV and cytokines will be considered here; CMV modulation of cytokine levels *in vivo* and *in vitro* and the effect of cytokines on CMV expression and replication.

CMV can induce increased cytokine production by both the infected cell and by cells such as leukocytes which interact with the infected cell. Table 3 shows the range of cytokines induced by infection of fibroblasts, many of which could contribute to immunopathology. For example, interferon (IFN)-β released from infected cells (Rodriguez *et al.*, 1987) can upregulate the expression of class I HLA on uninfected cells (Grundy *et al.*, 1988a). Another cytokine affected by CMV infection is interleukin (IL)-6 (Almeida *et al.*, 1994; St-Jeor *et al.*, 1993). Infected fibroblasts show induction of IL-6 expression and supernatants from CMV-infected cells induce IL-6 expression within 30 minutes of contact with endothelial cells (Almeida *et al.*, 1994).

Table 2.

Characteristic features of cytokines.

1. Most cytokines are simple polypeptides or glycoproteins with molecular weight =8-25 kD (but many cytokines form homodimers or homotrimers). One cytokine (IL-12) is a heterodimer.
2. Constitutive production of cytokines is usually low or absent; production is regulated by various inducing stimuli.
3. Cytokine production is transient and the action radius is usually short (typical action is autocrine or paracrine, not endocrine). However, some cytokines are found in the circulation during systemic infections.
4. Cytokines produce their actions by binding to specific high affinity cell surface receptors.
5. Most cytokine actions can be attributed to an altered pattern of gene expression in the target cells. Phenotypically, cytokine actions lead to an increase (or decrease) in the rate of cell proliferation, change in cell differentiation state, and/or a change in the expression of some differentiated functions.
6. Although the range of actions displayed by individual cytokines can be broad and diverse, at least some actions of each cytokine are targeted at hematopoietic cells.
7. Cytokines often act by increasing (or decreasing) or by trasmodulating receptors for other cytokines.
8. Structurally dissimilar cytokines may have a similar spectrum of actions (redundancy).
9. A single cytokine often has multiple target cells and multiple actions (pleiotropism).
10. Because cells and tissues in the body are rarely, if ever, exposed to a single cytokine at a time, many cytokine actions reflect the result of synergistic or antagonistic interactions among several cytokines.

Magnan *et al.* (1994) reported down-regulation of bone marrow stromal cell IL-6 production 72 h after CMV infection, even when cells were stimulated with lipopolysaccharide. However, Taichman *et al.* (1997) found no significant modification of IL-6 levels following infection of mixed bone marrow stromal and progenitor cells.

Table 3.

Cytokine induction by CMV.

Cytokines induced by CMV infection in cultured fibroblast cells
IFN-β
IL-6
TGF-β
Cytokines induced by CMV infection/challenge of monocytes/monocytic cell lines
TNF-α
IL-1
IFN-α

IL-6 is a cytokine which exerts multiple biological activities, including regulating immune responses, haematopoiesis. Among these, induction of T cell proliferation and cytotoxic T cell differentiation are just two of the many effects of IL-6 which may enhance pathological immune responses. In addition, IL-6 can affect cells in many different ways. When IL-6 binds to its receptors on B cells, the cells are stimulated to differentiate and secrete antibody (Hirano *et al.*, 1990). IL-6 like IFN-β , is an endogenous pyrogen (Mackowiak, 1998), and the viral induction of both of these cytokines could contribute to the high fever observed during episodes of CMV infection in transplant recipients (Lui *et al.*, 1992).

The production of transforming growth factor-β (TGF-β) is enhanced by CMV infection of fibroblasts (Michelson et al., 1994). CMV replication is

stimulated by treatment of fibroblast with recombinant TGF-β (Michelson *et al.*, 1994). A number of other cytokines have been reported to be produced by CMV infection or CMV challenge of monocytes or monocytic cell lines. Enhanced production of tumor necrosis factor (TNF)-α (Smith *et al.*, 1992; Turtinen *et al.*, 1989), IL-1 (Crump *et al.*, 1992; Iwamoto *et al.*, 1990), and IFN-α (Noraz *et al.*, 1997) have been described, all of which are endogenous pyrogens (Mackowiak, 1998) as well as pro-inflammatory cytokines.

1.6.3 Induction of cytokines by CMV in transplant recipients.

As concerns CMV and cytokines, two aspects will be reviewed. CMV modulation of cytokines *in vivo* and *in vitro* and the effect of cytokines on CMV expression and replication in transplant recipients.

1.6.3.1 Interleukins (ILs).

Interleukins are a large group of cytokines (IL-1 to IL-18) produced mainly by T cells, though some are also produced by mononuclear phagocytes, or by tissue cells. They have a variety of functions, but most of them are involved in directing other cells to divide and differentiate. Each interleukin acts on a specific, limited group of cells which express the correct receptors for that interleukin.

IL-1 is produced by macrophages and a variety of other cell types (Oppenheim *et al.*, 1986). The biological activities of the IL-1 molecules varied and important for immune reactivity. IL-1 can induce cells to synthesize more of itself and other cytokines, such as TNF, and IL-6. In fact, IL-1, IL-6 and TNF are all proinflammatory cytokines with overlapping biological properties. Another critical function of IL-1 is the activation of T cells (Herrmann *et al.*, 1988). As a consequence of this activation, IL-2, IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) are produced (Herrmann *et al.*, 1988).

Following CMV infection in liver transplant patients, IL-1 serum levels slowly decrease, whereas they remain elevated in patients undergoing acute

rejection or bacterial infections (Tilg *et al.*, 1991). In bone marrow transplant patients, IL-1 receptor antagonists serum levels are significantly increased in CMV-infected, CMV-reactivating patients, graft-versus-host disease or fever of unknown origin when compared with bone marrow transplant patients without complication (Schwaighofer *et al.*, 1997). Brain-derived macrophages and microglial cells do not produce IL-1 upon CMV infection (Pulliam *et al.*, 1995), suggesting cell type specificity in IL-1 induction by CMV. In contrast, infection of human endothelial cells in culture leads to suppression of IL-1 (by 82%) when assayed 5 hours postinfection (Woodroffe *et al.*, 1993).

IL-6 is another potent pleiotropic cytokine which is produced by a variety of lymphoid and nonlymphoid cells (Hirano *et al.*, 1990) (section 1.6.3). In lungs of CMV-infected lung transplant patients, there was a significant increase of IL-6 in sera and BAL fluid, compared to uninfected patients, patients without complications, and those undergoing acute rejection (Magnan *et al.*, 1994). A significant increase in IL-6 transcription in BAL fluid cells was also detected by *in situ* hybridization, suggesting that IL-6 was produced *in situ* in CMV-infected lungs (Humbert et al., 1993). The production of IL-6 by alveolar macrophages from lung transplant patients reflected *in vivo* measurements suggesting *in situ* production (Humbert *et al.*, 1993).

1.6.3.2 Interferons (IFNs).

Although the interferons were first identified in 1957 as antiviral proteins, they are now also recognised as immunoregulatory proteins capable of altering a variety of cellular processes, such as cell growth, differentation, gene transcription, and translation (Baron *et al.*, 1991; Hooks and Detrick, 1992). There are three general types of IFNs: IFN-α,-β, and -γ (Baron *et al.*, 1991). The cell making the IFN and the substance triggering its production are important factors in determining the type of IFN produced. IFN-α is produced by leukocytes in response to a variety of IFN inducers, such as viruses, bacterial products, polynucleotides, tumour cells, and allogeneic cells. The cell types responsible for

synthesizing IFN-α include B cells, T cells, macrophages, NK, and large granular cells. If any of these inducers should interact with fibroblasts, epithelial cells, or to a lesser extent, leukocytes, IFN-β is produced. As an integral part of the immune response, T cells (either $CD4^+$ or $CD8^+$) are capable of manufacturing IFN-γ. The interaction of sensitized T cells with antigens or specific antigen-antibody complexes results in the production of IFN-γ. The NK cell has also been shown to produce IFN-γ. Moreover, the cytokine IL-2, which is produced by T lymphocytes, can also stimulate T cells to produce IFN-γ.

The IFNs can positively or negatively regulate cellular actions. These IFNs may then function in a number of ways: they may inhibit virus replication, suppress cell proliferation and oncogene expression, and alter cell differentation. The antiviral actions of the IFNs are not based on their direct interaction with virions. IFNs can modify immune reactivity by acting at the level of B cells, T cells, NK cells, macrophages, basophils, or bone marrow stem cells (Baron *et al.*, 1991; Hooks and Detrick 1992).

CMV not only induces interferon (Rodrigues *et al.*, 1987) but is sensitive to its action *in vitro* (Rasmussen *et al.*, 1984), although its role *in vivo* is unknown. No mechanisms are currently known by which the virus could evade this potentially protective host anti-viral response.

Levels of IFN-α were not notably increased in sera of CMV-infected liver transplant recipients (Arnold *et al.*, 1993). Feldman *et al.*, (1994) studied the frequency of IFN-α -producing cells following *in vitro* challenge of peripheral blood mononuclear cells with DNA and RNA viruses. Enveloped viruses such as CMV elicited an IFN-α response.

IFN-β is the main form of IFN induced by CMV infection of endothelial cells (Sedmak *et al.*, 1995). UV-irradiated CMV-infected cell supernatants partially block MHC class II expression in uninfected cells. This block can be partially alleviated by the addition of anti-IFN-β antibodies, suggesting that CMV-induced IFN-β plays some role in CMV-induced inhibition of MHC class II expression (Sedmak *et al.*, 1995). Low-multiplicity infection of fibroblasts with CMV

apparently induces IFN-β production, which can protect surrounding cells from infection (Hamprecht and Steinmassl, 1994).

IFN-γ does not appear to be elevated in BAL fluid of bone marrow transplant patients with CMV pneumonia (Slavin *et al.*, 1993). Human CMV IE-specific $CD4^+$ T cell clones, when stimulated specifically *in vitro*, secrete IFN-γ in quantities sufficient to inhibit CMV replication in neighboring cells (Davignon *et al.*, 1996). Co-culture of activated lymphocytes from peripheral blood of bone marrow transplant recipients, with autologous CMV-infected fibroblast greatly augmented the production of IFN-γ (Duncombe *et al.*, 1990). The production of this cytokine has also been described following culture of $CD4^+$ T cells with infected endothelial cells (Waldman and Knight, 1996).

Treatment of human fibroblasts with IFN-α or IFN-β hardly affects IE protein synthesis but blocks early and late antigen expression, as well as extracellular virus production up to 99% (Torigoe *et al.*, 1993). In this respect, there is one case report of successful treatment with IFN-β of a renal allograft patient who had CMV-associated hepatitis and duodenal ulcer (Yagisawa *et al.*, 1995).

1.6.3.3 Tumor necrosis factors (TNFs).

TNFs are cytokines with inflammatory and antitumor activity which are presently identified as TNF-α and TNF-β. TNF-α and TNF-β are structurally related, binds to the same cellular receptors and produce similar biological changes in a variety of cells. While TNF-α is produced by neutrophils, activated lymphocytes, macrophages, NK cells, and some nonlymphoid cells, such as astrocytes, endothelial cells, smooth muscle cells, and some transformed cells, TNF-β appears to be solely produced by T cells.

CMV infection is associated with a significant elevation of TNF-α in bronchoalveolar (BAL) fluid in lungs of lung transplant recipients, compared to what is seen in the lungs of patients undergoing only acute rejection (Rondeau et

al., 1991; Smith et al., 1992). TNF is not increased in sera of latter patients but TNF receptors are elevated in the lungs of pulmonary transplant patients (Humbert *et al.*, 1994).

CMV IE protein-specific human $CD4^+$ T cell clones stimulated with antigen produce TNF-β in amounts which play a role in inhibiting CMV replication in neighbouring astrocytoma cells (Davignon *et al.*, 1996). Docke *et al.*, (1994); and Fietze *et al.*, (1994) proposed that enhanced TNF levels observed during various diseases might be a factor for enhancing CMV replication *in vivo*. They tried to establish a correlation between CMV antigenemia and enhanced TNF levels in plasma and found that blood mononuclear cells of almost all patients with septic disease were positive for CMV antigen and that corresponding plasma contained enhanced levels of TNF.

The interaction between lymphocytes and CMV-infected cells such as fibroblasts can augment cytokine production from lymphocytes. This has been shown for '*in vitro* activated' lymphocytes from peripheral blood of bone marrow transplant recipients, where co-culture with autologous CMV-infected, but not uninfected, bone marrow fibroblast greatly augmented the production of the pro-inflammatory cytokine TNF-α (Duncombe *et al.*, 1990). The production of this cytokine has also been described following culture of $CD4^+$ T cells with infected endothelial cells (Waldman and Knight, 1996). CMV-induced TNF production can enhance CMV expression. TNF, via interaction with either of its receptors (p75 and p55) upregulates CMV replication. (Laegreid *et al.*, 1994; Medvedev *et al.*, 1996). The results imply that stimulation of either of the TNF receptors can generate intracellular signals leading to induction of factors that can stimulate transcription from CMV genome (Medvedev *et al.*, 1996).

1.6.3.4 Transforming growth factor β.

TGF-β is a multifunctional cytokine that often demonstrates opposing effects, on the development, physiology, and immunologic responses of a variety

of cells. This cytokine is known to influence cellular differentiation, bone formation, haematopoiesis, cell cycle progression and cell migration.

TGF-β belongs to a family of proteins that can potently down-regulate lymphocyte functions (Kehrl *et al.*, 1991; Sporn and Roberts, 1992). Through this mechanism, it plays an important role in modifying inflammatory and immunological responses (Wahl *et al.*, 1989). Only one study has addressed TGF-β levels *in vivo* in the supernatants of alveolar macrophages from lung transplant patients with CMV infection. This showed that cellular TGF-β content increased but not TGF-β secretion (Magnan *et al.*, 1996).

In vitro studies show that CMV infection of fibroblast induces transcription and secretion of TGF-β (Michelson *et al.*, 1994). Following infection of bone marrow stromal cells stimulated with lipopolysaccharide, two patterns of TGF-β production were seen: either decreased production irrespective of lipopolysaccharide stimulation or no effect of CMV (Lagneaux *et al.*, 1996; Taichman *et al.*, 1997).

1.6.4 Concluding remarks.

CMV maintains an intriguing and complex relationship with its host. It establishes latency in cells involved in immune responses, particularly bone marrow progenitors and monocytes. Replication of CMV in macrophages has been shown to depend on the differentiation of these cells (Ibanez *et al.*, 1991; Lathey and Spector, 1991). Thus when CMV is threatened by an immune response, it is precisely this response that activates CMV replication. Subsequently, its replication has negative effects on these and surrounding cells, thereby rendering the immune response incapable of eliminating the virus completely. CMV also seems to take advantage of soluble mediators of immune responses, either by sequestering them, or by using their stimulatory effects on certain pathways to replicate more efficiently. Thus, CMV has established a symbiotic relationship with its host, thereby assuring its perpetuation.

1.7 MAJOR OBJECTIVES AND AIMS.

In view of the importance of CMV infection in kidney transplant recipients this study has two major objectives. The first objective is to establish flow cytometric assay for the detection of the CMV related pp65 antigen in PMNL of kidney transplant recipients since flow cytometry is generally highly sensitive, quantitative, rapid and objective. The second objective is to investigate the cell-mediated immune response in CMV-infected kidney transplant recipients by detecting the level of various cytokines and by analyzing different leukocyte subsets for any alteration in numbers.

The first objective involves the establishment of a flow cytometric assay for the early detection of CMV-specific antigens in PMNL by using monoclonal antibodies. Then, a comparison between CMV-antigenemia and flow cytometric assays for the detection of CMV-specific antigen (pp65) will be made.

The second objective involves investigating peripheral blood mononuclear cells (PBMC) from CMV-infected and CMV-uninfected kidney transplant patients for cellular responses to the mitogen (PHA) and CMV antigens. On the other hand, levels of Th1-type cytokines (IL-2, TNF-α, IFN-γ) and Th2-type cytokines (IL-4, IL-10) will be measured in the supernatant after coculturing PBMC of CMV-infected and CMV-uninfected kidney transplant patients with PHA and CMV antigen. Also, flow cytometric analysis will be made for different lymphocyte subsets from CMV-infected and CMV-uninfected kidney transplant patients for the detection of alterations, if any in numbers of T lymphocytes, T cell subsets, B cells, granulocytes and NK cells.

2. MATERIALS, METHODS AND STUDY POPULATION.

2.1. Blood samples.

Pretransplant blood samples were collected from recipients before the administration of immunosuppressive drugs. Post-transplant samples were collected every two weeks for a period of 6 months. Blood samples were also collected during deterioration of renal function and at any suspected CMV-related infections in two EDTA containing tubes and transferred to the laboratory within 2-3 hours. One tube was processed for antigen detection and the second tube was processed for mitogen and CMV-induced stimulation of PBMC.

2.2. Study population.

2.2.1 General characteristics.

Table 4 shows, some demographic parameters of the study population. Seventy-nine kidney transplant patients (31 female, 48 male) who received kidney transplants between 1996 and 1998 were inducted into this study.

Table 4

Demographic parameters of the study population.

Mean Age (range)	Sex		Ethnic origin	
	M (%)	F (%)	Kuwaiti (%)	Non-Kuwaiti (%)
37 (18-60)	48 (61%)	31 (39%)	73 (92%)	6 (8%)

* IgG test was done before transplantation.

As can be seen from table 4, the age range is from 18 to 60 years, with an average of 37 years. Seventy-three kidney transplant patients were Kuwaiti and 6 were Non-Kuwaiti (2 Egyptians, 2 Indians, 1 Syrian, 1 Iranian). All patients received an immunosuppressive regimen of cyclosporin A, azathioprine and steroids. Post

transplantation patients received a fixed amount of cyclosporin as specified by Table 5. Cyclosporin TRAF levels were checked in whole blood every 12 hours.

Table 5

Cyclosporin level recommended for kidney transplant patients.

Time after transplantation	Blood cyclosporin level ng/ml
0-3 weeks	250-300
1-3 months	200-250
4-6 months	150-200
6-12 months	100-150

2.2.2 Specific data.

2.2.2.1 CMV seropositivity.

CMV-IgG was determined on pretransplant blood sample by indirect immunofluorescent test (Gull Laboratories, USA). Only one patient was IgG negative.

2.2.2.2 Underlying disease.

The underling disease complications that lead to kidney loss are categorised as folllows: glomerulonephritis (n=31), chronic tubulointerstitial disease (n=13), diabetes mellitus (n=10), adult polycystic kidney disease (n=9), hypertension (n=6), obstructive uropathy (n=5) and other causes (n=5).

2.2.2.3 HLA matching.

HLA matching was done on three molecules; HLA-A, HLA-B, and HLA-DR. 12 recipients had a complete match, 8 with one allele mismatch, 27 with two allele mismatch, 10 with three allele mismatch, 12 with 4 alleles mismatch, and 10 with 5 alleles mismatch. HLA matching of the recipients is shown in Table 6.

Table 6

Allele differences between donors and recipients

	Complete match	Differences in alleles				
		1	2	3	4	5
No of recipients	12	8	27	10	12	10

2.2.2.4 Source of donor kidney.

Due to the shortage of live related or unrelated donor kidney, almost half of the patients received cadaver kidneys. The other half of the recipients received live related or un-related kidneys (Table 7).

Table 7

Source of donor kidney.

Donor			Total
Live related	Live unrelated	Cadaver	
20 (25%)	26(32%)	33 (42%)	79

2.2.3 Diagnostic criteria.

In principle diagnosis of CMV infection/disease conformed to the guidelines suggested by Ljungman and Plotkin (1995). Clinically, a CMV infection was suspected when recipients suffered one of the following symptoms: unexpected fever, arthralgia, leukopenia, thrombocytopenia, pneumonitis, hepatitis, retinitis and gastrointestinal ulceration. Patients who had at least one of the CMV-associated symptoms and being antigenemia –positives were considered to have a CMV disease.

2.3 Cell lines.

The MRC-5 diploid cell line-derived from fetal lung tissue was obtained from the American Type Culture Collection (ATCC) and maintained in the growth medium described below.

2.4 Human cytomegalovirus (CMV).

The AD 169 strain of human cytomegalovirus was obtained from ATCC.

2.5 Growth and maintenance media.

Minimal essential medium (MEM) and RPMI-1640 medium were purchased from GibcoLife Sciences, UK. In order to prevent microbial contamination the following antibiotics (GibcoLife Sciences, UK) were added to the media: fungizone at a concentration of 50 units/ml of medium, penicillin at a final concentration of 100 units/ml, and streptomycin sulfate at a concentration of 100 μg/ml of medium. Growth and maintenance media were supplemented with 10% fetal bovine serum respectively (FBS; Flow Laboratories, UK) for the growth medium or 2% FBS for the maintenance medium.

2.6 Trypsin versene solution (T/V).

Trypsin-versene (T/V) solution consisted of 0.25% trypsin (GibcoLife Sciences, UK) in phosphate buffer saline (PBS) containing 0.02% ethylene diamine tetraacetic acid (EDTA; GibcoLife Sciences, UK). PBS, magnesium and calcium free phosphate buffered saline in tablet form were purchased from Flow Laboratories, UK. Ten tablets were dissolved in one liter of distilled water and autoclaved. The solution was then stored at 4°C.

2.7 Cell culture preparation.

Cell monolayers grown in Roux flasks (25 cm^2 or 75 cm^2 flasks, Nunclon, Denmark) were split and propagated according to Hsiung (1982). The medium was discarded from the flask and the cell layer was washed two to three times with PBS. Prewarmed T/V was added to the flask (1 ml to 25 cm^2 flask and 2 ml to 75 cm^2 flask). After one minute, the excess T/V was decanted and the flask was incubated at 37°C for another 5-10 minutes, if needed until the cells detached. An adequate amount of prewarmed MEM growth medium was added to the flask and the cells were suspended by pipetting to break any clumps which might be present within the cell suspension. Flasks of 25 cm^2 and 75 cm^2 were then seeded with 10 ml and 15 ml of cell suspension, respectively. They were then incubated at 37°C until further use. For 96-well plates, 0.1 to 0.2 ml of cell suspension was added to each well (10,000 cells/well).

2.8 Virus titration.

Virus titration was performed as described by Grist *et al.*, (1974). An aliquote of stock virus suspension was thawed at 37°C, and ten-fold serial dilutions were made in MEM (e.g. from 10^{-1} to 10^{-8} dilutions). Virus titrations were carried out in 96-well tissue culture plates (flat-bottomed 96-well, Falcon products, USA). When a confluent monolayer developed in the wells, medium was removed and the

cell layers were washed three times with PBS. Each virus dilution was inoculated (0.1 ml/well) in duplicate wells. After a 30 minute incubation for virus adsorption, 0.1 ml of maintenance medium was added to each well. Control cells were maintained in 0.2 ml of maintenance medium. Plates were then incubated at 37°C and the degree of CPE was recorded daily microscopically. The titre of the virus was then calculated by the 50% tissue culture infectious dose end point ($TCID_{50}$) method of Reed –Muench (1938).

2.9 Propagation of virus in cell culture.

The human CMV strain AD169 was propagated in MRC-5 cell monolayers grown in 25 cm^2 or 75 cm^2 flasks at one time and the same batch of CMV-infected MRC-5 was used throughout the study. The medium was discarded from the flask and the monolayer was washed two or three times in prewarmed PBS. The cell layer was incubated with 0.3 ml of stock virus. The multiplicity of infection was 0.1 virus/cell. After a 30 minute incubation at room temperature maintenance medium was added. Flasks were then incubated at 37°C and observed daily for the appearance of cytopathic effect (CPE). When the CPE progressed to 100%, medium was collected and any detached cells were removed by centrifugation for 10 minutes at 1500 rpm. Undetached cells were removed by trypsinization (section 2.4). After centrifugation the pellets were collected, suspended in MEM medium and stored at -70°C in aliqoutes of 10^6 cells/ml. The count was adjusted by using a haemocytometer (Neubauer, W.Germany).

2.10 CMV Antigenemia assay (AA).

2.10.1 Specimen Collection and Preparation.

Approximately 5 ml of venous EDTA-treated venous blood was collected from each patient on using aseptic venipuncture technique. Samples collected were processed for the AA within 2-3 hours.

2.10.2 Isolation of leukocytes from peripheral blood and immunoperoxidase staining.

Polymorphonuclear leukocytes (PMNL) were isolated by the dextran sedimentation method as previously described (Grefte *et al.*, 1992a,b). Following incubation and centrifugation, the cell pellet was suspended in PBS and erythrocytes lysed with 0.8 mM ammonium chloride. The remaining cells were centrifuged, washed in PBS, counted by a coulter counter (Coulter Electronics, USA) and spotted onto CMV-vue microscopic slides (INCSTAR Corporation, USA) (50,000 cells per spot). Each microscopic slide contained one positive control well consisting of fibroblasts infected with CMV strain AD169, one negative control well consisting of uninfected fibroblasts. They were then dried and fixed in acetone, stained with CMV-vue Kit (INCSTAR Corporation, USA) according to the manufacturer's recommendation. PBML were incubated with a mixture of two different monoclonal antibodies (clones C10 and C11) directed against the CMV lower matrix, early structural protein. PMNL containing the CMV-specific pp65 antigen were counted under a light microscope.

A dark brown to red-brown nuclear or perinuclear staining within PMNL was taken as a positive result. Samples from each patient were tested in duplicate and scanned at 200x-400x magnification for the presence of CMV-infected cells. A sample displaying 5 or more pp65 antigen-containing cells out of 50,000 was considered as being positive. A lack of specific staining of PMNL upon examination of the duplicate wells was taken as a negative result. Any cytoplasmic colouration in the absence of nuclear or perinuclear staining was considered negative.

2.11 Stimulation of PBMC with PHA.

2.11.1 Purification of PBMC.

Peripheral blood mononuclear cells (PBMC) of AA-negative and AA-positive kidney transplant recipients were obtained by Ficoll-Hypaque (Pharmacia Biotech, Sweden) density gradient centrifugation of 5 ml of peripheral blood. Blood

was carefully layered over Ficoll-Hypaque solution in a test tube, in a ratio of 2:1 (5 ml blood, 2.5 ml Ficoll-Hypaque solution). Care was taken to avoid mixing of blood with the gradient. The sample was centrifuged at 1800 rpm at room temperature for 15 minutes. The interface, which contained the PBMC, was carefully removed with a Pasteur pipette and washed 3 times in a test tube using RPMI-1640 medium containing 10% FBS. Washing was done by centrifugation at 1200 rpm for 10 minutes after which the supernatant was discarded. The pellet containing the PBMC was suspended and counted by using a Coulter counter.

2.11.2 PHA-induced proliferation of PBMC.

PBMC suspended in RPMI-1640 medium and adjusted to 10^6 cells/ml aliquoted into 96 well tissue culture plates at a density of 10^5 cells in 100 µl per well, and 100 µl phytohemagglutinin (PHA) (Sigma Chemicals, USA) of a 5 µg/ml solution was added for a period of 24 and 96 hours at 37°C. The concentration of 5 µg/ml was chosen on the basis of optimal proliferation in our laboratory. For each sample a negative control was included by incubating PBMC for the same period in the absence of PHA. The supernatants from three wells were harvested after 24 hours and six wells after 96 hours, while cells in an another three wells were pulsed with 1 µCi per well [^{3}H] thymidine (Amersham, U.K.) at 96 hours of incubation for assessment of PHA-induced proliferation. Thymidine-pulsed wells were harvested 18 hours later onto glass filter fibers (1205-401, Filtermat A, Wallac, Turku, Finland) and the radioactivity was counted by a Betaplate 1205 Scintillation counter (Pharmacia, Finland). The stimulation index was calculated as a ratio of thymidine uptake between PHA-stimulated PBMC and non-stimulated PBMC. Background thymidine uptake in the absence of PHA was in the range of 80 cpm to 500 cpm. Stimulation indices of 50 or more were considered positive. The cut-off of 50 was based on experience in our Clinical Immunology Laboratory that an S.I. less than 50 generally indicates low proliferation due to poor immune status of the subjects.

2.11.3 CMV-induced proliferation of PBMC.

PBMC of AA-positive and AA-negative kidney transplant recipients and CMV-infected fibroblast were suspended in RPMI-1640 medium and adjusted to 10^6 cells/ml aliquoted into 96 well tissue culture plates at a density of 10^5 cells in 100 µl per well with 100 µl of a 10^5 freeze-thawed CMV-infected fibroblast per well and incubated for a period of 24 and 96 hours. The concentration of 10^5 CMV-infected fibroblast per well was chosen on the basis of optimal proliferation. For each sample a negative control was included by incubating PBMC for the same period of time with uninfected fibroblasts. The supernatants from three wells were harvested after 24 hours and six wells after 96 hours, while cells in an another three wells were pulsed with 1 µCi per well [^{3}H] thymidine (Amersham, U.K.) at 96 hours of incubation for assessment of CMV-induced proliferation. Thymidine-pulsed wells were harvested 18 hours later onto glass filter fibers (1205-401, Filtermat A, Wallac, Turku, Finland) and the radioactivity was counted by a Betaplate 1205 Scintillation counter (Pharmacia). The stimulation index was calculated as a ratio of thymidine uptake between CMV-stimulated PBMC and uninfected fibroblast-stimulated PBMC. Background thymidine uptake of PBMC stimulated with uninfected fibroblast control was in the range of 100 to 1000 cpm. Stimulation indices of less than 3 were considered to be proliferation negative (Boland *et al.*, 1990; van Zanten *et al.*, 1995).

2.12 Assay for cytokines.

Th1-type cytokines (IL-2, TNF-α and IFN-γ) and Th2-type cytokines (IL-4 and IL-10) were measured by ELISA using kits obtained from Immunotech SA (France). The recommendations of the manufacturer were followed.

2.12.1 Assay principle.

Supernatants were assayed by sandwich ELISA with two immunological steps. The standard and sample were assayed at the same time for each run. Briefly, the first step of the procedure leads to the capture of the relevant cytokines (present in the sample) by monoclonal anti-cytokine antibodies bound to the wells of microtiter plates. In the second step, biotinylated monoclonal antibody was added together with streptavidin-enzyme (peroxidase or alkaline phosphatase) conjugate. The biotinylated antibody binds to the antibody-antigen complex, and in turn, binds the conjugate. After incubation, the wells were washed and the binding of streptavidin-enzyme via biotin was detected by the addition of a chromogenic substrate. The intensity of the colour produced was proportional to the concentration of the cytokine present in the sample.

2.12.2 Assay procedure.

Cytokines were estimated in the supernatants of PBMC of AA-negative kidney recipients and AA-positive recipients stimulated with PHA and CMV-infected fibroblasts for 24 hours (for IL-2) and 96 hours (for TNF-α, IFN-γ, IL-4 and IL-10). The lyophilized cytokine standard was recostituted as recommended. Serial dilutions of the cytokine standard were prepared prior to each assay. Two well on the plate was left empty for subtrate blank. 50 µl per well of standard or sample were added in duplicate and incubated for 2 hours at room temperature (18-25°C) with shaking (350 rpm). The first immunological step was followed by washing the wells, including those of substrate blank, using an automatic washer (Labsystems, Finland). After washing the plate for three times, 50 µl of biotinylated antibody and 100 µl of streptavidin- conjugate were added to all wells, except to that for standard blank and incubated for 30 minutes at room temperature (18-25°C) with shaking (350 rpm). The second immunological step was followed by washing the wells, including that of substrate blank, as before. Then 100 µl of chromogenic substrate was added to all wells, including that for substrate blank. After that the plate was

incubated for 30 minutes at room temperature (18-25°C) in the dark with shaking (350 rpm) . Finally, 50 µl of stop solution was added to all wells, including the subtrate blank and the plate was read at 450 nm against substrate blank. The colored product was measured using a Multiscan MS microplate reader (Labysystems, Finland). The sensitivity of each of the assays was as follows: 5 pg/ml for IL-2, 10 pg/ml for TNF- α, 3 pg/ml for IFN- γ, 5 pg/ml for IL-4 and 5 pg/ml for IL-10.

2.12.3 Evaluation.

The concentration of cytokines in a sample was determined by interpolation from a standard curve that was constructed for every test run using reference recombinant cytokines. The standard curves were drawn on linear graph paper, plotting the concentrations of the cytokine standard on the horizontal axis and the corresponding average absorbance on the vertical axis. The "spline" mode was used for a computerized evaluation of the results. The absorbance of the unknown was plotted on the vertical axis and was read off the corresponding concentrations of the samples cytokines on the horizontal axis.

2.13 Flow cytometry.

2.13.1 Fixation of CMV-infected cell cultures for flow cytometry.

As described before (section 2.7), MRC-5 cells were inoculated with stock virus and after 30 minutes absorption period fresh medium was added, and the incubation started. This was considered 0 time.

Detached cells were collected by centrifugation at 1500 rpm for 10 minutes. Attached cells were removed with a 0.25% T/V solution, centrifuged at 1500 rpm for 10 minutes, added to detached cells, suspended in PBS, counted with a haemocytometer (Neubauer, W.Germany), adjusted to 1.0 -1.5 x 10^6 cells/ml and fixed in cold 90% methanol (1 part PBS + 9 parts MEOH) with continuous shaking and stored at -20°C until processed for flow cytometric analysis (Elmendorf et al., 1988; Mcsharry, 1994).

2.13.2 Collection of granulocytes for flow cytometric analysis.

The dextran sedimentation method described before (section 2.8.2) was used to isolate PMNL from the blood samples. Then PMNL were counted (Coulter counter, USA) and adjusted to $2x10^6$ cells/ml with cold PBS, fixed with cold 90% methanol and stored at -20°C until they were stained for flow cytometric analysis.

2.13.3 Staining of PMNLs and MRC-5 for Flow cytometric analysis.

Fixed and permealized cells (MRC-5 or PMNL) were stained by the procedure of Impert-Marcille *et al.*, (1997) using mouse monoclonal antibodies (Clonab, Biotest, Dreieich, Germany), specific to the CMV lower matrix, early structural (pp65) antigen. After removing the fixative by centrifugation at 1000 g for 5 minutes, cells were washed in PBS containing 20% human AB serum (PBS/AB). Cells were resuspended in 200 µl of mouse monoclonal antibody (Dako, Denmark) to CMV pp65 antigen diluted 1:5 in PBS/AB. Optimal staining was achieved at 1:5 dilution of mouse monoclonal antibody to CMV pp65 antigen. For each test sample, a control was included. Thus, control cells were suspended in 200 µl of isotypically-matched monoclonal antibody (IgG2b) (Caltag/Tebu, Marnes la Coquette, France). Both tubes were then incubated at 37°C for 60 minutes with shaking. The cells were washed twice with PBS/AB by centrifugation and then both tubes sample and control were incubated with 200 µl of 1:20 diluted FITC-conjugated goat anti-mouse immunoglobulin (Clonab, Biotest, Dreieich, Germany) at 37°C for 60 minutes with shaking. Optimal staining was achieved at 1:20 dilution of FITC-conjugated goat anti-mouse immunoglobulin. This was followed by two rinses in PBS/AB and the cells were suspended in 200 µl of PBS/AB and analyzed by an EPICS-Profile II (Coulter Electronics, USA) flow cytometer.

Controls used: mock-infected MRC-5 fibroblast cells, PMNL from patients without active CMV infections served as negative controls. MRC-5 cells infected

with the AD169 reference strain (at a multiplicity of infection between 0.1 virus/cell) served as positive control.

2.14 Immunofluorescence cell surface staining with monoclonal antibodies.

2.14.1 Staining procedure for surface markers.

The assay was performed within 5 hours of venipuncture. Blood samples were kept around 25°C until processing. The following monoclonal antibodies (Cyto-Stat/Coulter Clone, Coulter electronics, USA) were used:

1. Double labeled monoclonal antibodies:

i) Clone T4-RD1/T8-FITC: recognizes $CD4^+$ (T cell inducer) and $CD8^+$ (T cell suppressor/cytotoxic) lymphocytes.

ii) Clone T11-RD1/B4-FITC: recognizes $CD2^+$ (pan T cell) and $CD19^+$ (pan B cell) lymphocytes.

iii) Clone CD3 FITC/CD16/CD56 RD1: recignizes $CD3^+$ (mature T cells), $CD16^+$ (NK cells, minority of T cell, granulocytes and some macrophages) and $CD56^+$ (NK cells and minority of T cell).

2. Single-labeled monoclonal antibodies

i) Clone CD15-FITC: recognizes $CD15^+$ (granulocytes) in whole blood by flow cytometry.

ii) Clone Ta1-FITC: recognizes $CD26^+$ (activated T lymphocytes, B lymphocytes and macrophages) in whole blood by flow cytometry.

Appropriate isotype controls were used for the monoclonal antibody optimal staining was achieved with white blood cell counts in the range of 3-10 x 10^6 cells/ml. White blood cell counts exceeding 10 x 10^6 cells/ml required dilution, while counts below 3 x 10^6 cell/ml required centrifugation and resuspension in autologous plasma, to achieve counts in the range of 3-10 x 10^6 cells/ml.

For each sample, eight siliconized 12 x 75 mm tubes were used. Five contained specific monoclonal antibodies and the remaining three served as isotype controls. Ten µl of monoclonal antibodies and isotype control monoclonal antibodies were added to the labeled tubes. Then 100 µl of the venous blood sample was added to each test tube and vortexed gently. Samples were processed after an incubation period of 10-12 minutes at room temperature (18-25°C).

2.14.2 Preparation of Leukocytes.

Leukocytes were prepared using Coulter Immunoprep Leukocyte System which is a rapid, gentle, no-wash erythrocyte-lysing system that maintains the integrity of leukocyte morphology and cell surface. After the staining procedure, test tubes were placed into Coulter Q-prep Immunology workstation. The reagents delivered to the blood sample included:

i) Red blood cell lysing agent (Formic acid) 600 µl/sample

ii) Stabilizing agent (sodium carbonate, sodium chloride and sodium sulphate) 265 µl/sample

iii) Cell membrane fixative (paraformaldehyde) 100 µl/sample

Prepared samples were kept at room temperature and analyzed by flow cytometry within two hours.

2.14.3 Flow cytometric analysis.

Samples were analyzed by an EPICS-Profile II flow cytometer. Fluorescence intensity of the cells was measured with a 488-nm air-cooled Argon laser in the flow cytometer. The instrument was calibrated daily by using various quality control reagents. Data acquisition was triggered by cell size (forward versus 90 light scatter). The green fluorescence was filtered through a 530 band pass absorption filter and red fluorescence was filtered through a 575 band pass absorption filter. Debris and dead cells were excluded from the analysis by the conventional scatter gating method. At least 50,000 PMNL or 10,000 MRC-5 fibroblasts were gated by

light scatter (PMNL gates were assessed for accuracy when appropriate by using CD15 staining. All data were expressed in log fluorescence histogram form. The threshold of positivity for the green or red fluorescence intensity was arbitrarily set based on the negative control sample.

2.15 Statistical analysis.

For calculating the cell count by heamocytometer the following formula was used: Cell count (per ml) = number of cells counted/number of $1mm^2$ squares counted x 1/dilution factor x 10^4 cell/ml.

For calculating the positive predictive value (PosPV) and the negative predictive value (NegPV) the following formulas were used: PosPV = (number of patients with positive test results and CMV-related disease/total patients with positive test results) x100. NegPV = (number of patients with negative test result and without CMV-related symptoms/total patients with negative test results) x100.

Cytokine data were analyzed by standard non-parametric t-tests. The *p* values were derived from standard Mann-Whitney tests using the SPSS statistics software package (SPSS Inc, New York, USA).

3. RESULTS

3.1 Flow cytometric assay.

3.1.1 Introduction.

It is of great importance to diagnose active CMV infection as early as possible using a rapid, sensitive and accurate diagnostic procedure to avoid over-treatment with immunosuppressive drugs and to guide antiviral therapy. During active CMV infection, PMNL appear in the circulation in low frequencies that express the CMV pp65 protein antigen. Because flow cytometry is an advanced technology, which can be used to analyze a large number of specifically labeled cells in a quantitative manner, it seemed appropriate to use flow cytometry for rapid and accurate detection of CMV-specific antigens in leukocytes of clinical samples of CMV-infected patients. This early detection of CMV-infected cells would then provide a reliable methology for the diagnosis of CMV infection especially in kidney transplant patients. In view of this, my first objective was to establish a flow cytometric assay for the early detection of CMV-specific antigens in PMNL.

3.1.2 Establishment of a model cell culture system for standardizing flow cytometric detection of CMV-specific antigens.

Prior to the detection of CMV-specific antigens in PMNL in clinical samples from kidney transplant recipients it was essential to be able not only to detect CMV-specific pp65 antigen in CMV-infected MRC-5 cells but also to distinguish between CMV-infected and uninfected MRC-5 cells by flow cytometric assay (FCA). For the purpose of standardization, MRC-5 diploid cell line was inoculated with AD169 strain of CMV (multiplicity of infection = 0.1 virus/cell) and incubated for seven days (section 2.6). CMV-infected and uninfected MRC-5 cells were fixed (section 2.11.1) and stained with monoclonal antibody to CMV pp65 antigen (section 2.11.3). Optimal staining was achieved at 1:5 dilution of monoclonal antibody to CMV pp65 antigen. Mock-infected MRC-5 cells were used as negative control to

exclude nonspecific fluorescence. FCA was carried out in duplicate and repeated three times to ensure validity of the data. When infected MRC-5 diploid cell line was tested for the presence of pp65 antigen by flow cytometry it was possible to detect the presence of antigen presenting cells as early as one day post infection. Figure 3 show the results of the FCA for the detection of CMV-specific pp65 antigen in CMV-infected MRC-5 cells from day one to day seven post infection. The percentage of cells expressing the pp65 antigen increased proportionally with time after infection. On day one post infection more than 51% of the cells expressed the antigen. That was followed by a gradual increase until it reached more than 90% on day seven (Fig. 3). It was important to know whether FCA seperates between CMV-infected and uninfected MRC-5 cells. How FCA differentiates between infected and non-infected cells can be demonstrated the best by histograms. As it is presented in Fig. 4 the fluorescence histogram of FCA gives a complete seperation between CMV-infected and uninfected MRC-5 cells.

The results of FCA obtained with CMV-infected and uninfected MRC-5 cells were encouraging to study the usefulness of flow cytometry for the detection of CMV-specific pp65 antigen in PMNL of kidney transplant recipients.

3.1.3 Detection of CMV-specific pp65 antigen in PMNL by antigenemia and flow cytometry assays.

Following the standardization of FCA using CMV-infected MRC-5 cells, the assay was tested on PBMLs of kidney transplant recipients with and without active CMV infection. Since the CMV antigenemia assay has been used in our laboratory for the early diagnosis of CMV infection in kidney transplant recipients, it provided an opportunity to compare the validity of FCA with that of the antigenemia assay. For this purpose, PMNL were separated from blood samples using the dextran sedimentation method (section 2.8.2). Isolated PMNL from each clinical sample were tested by both assays as described for the FCA in section 2.11.1 and 2.11.3 and for the antigenemia assay (AA) in section 2.8.8.

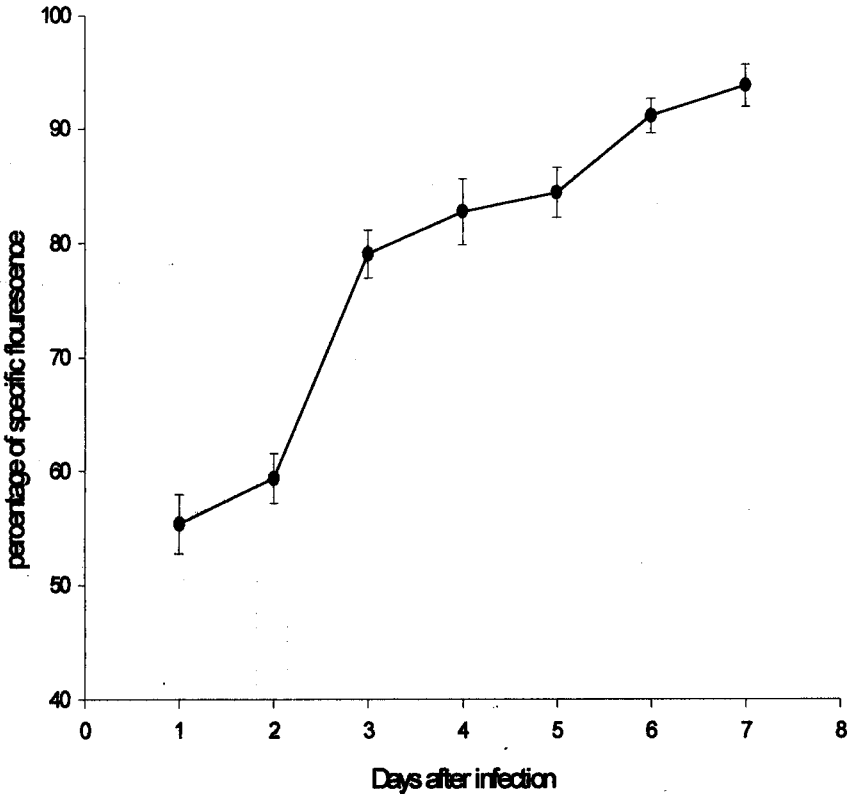


Fig. 3. Detection of pp65 antigen in CMV-infected MRC-5 cells. CMV-infected MRC-5 cells were harvested at daily intervals from day one to day seven post infection and screened using flow cytometry to detect the proportion of cells containing the pp65 antigen. Closed circles represent the mean percentage (±SEM) of the specific fluorescence.

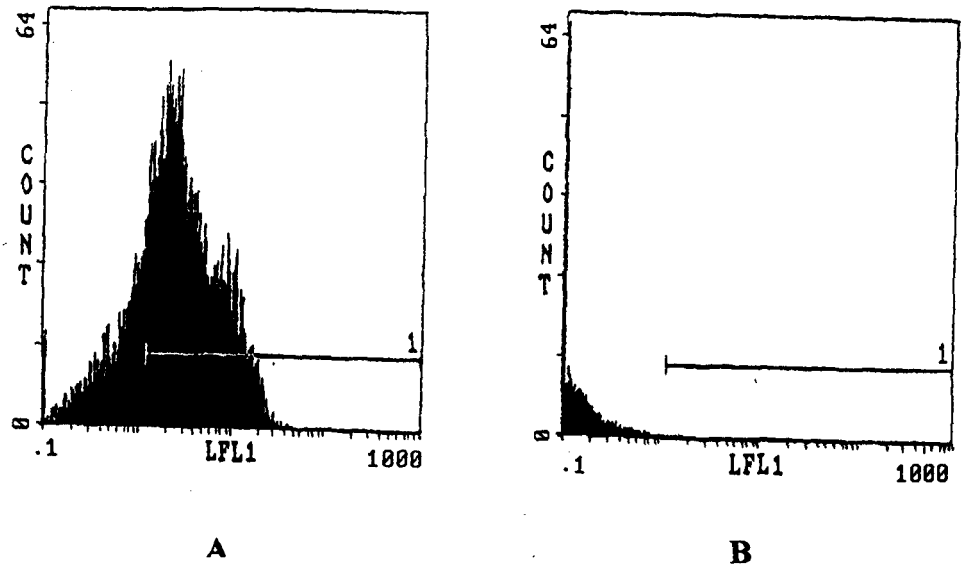
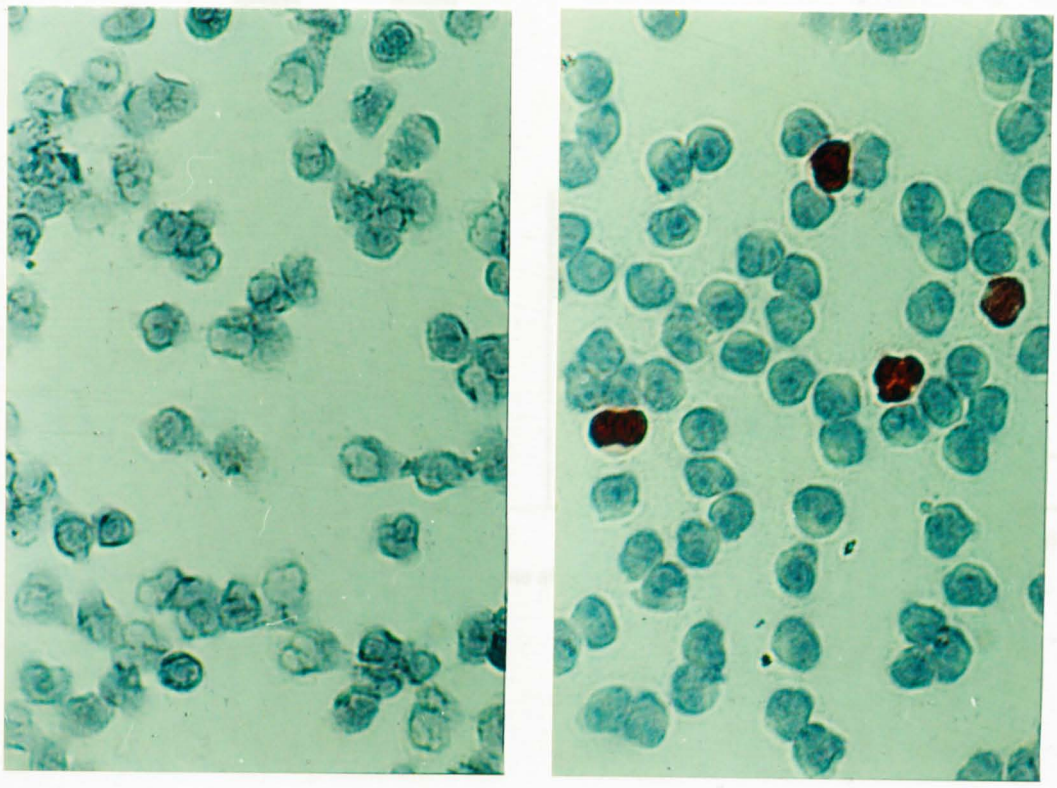


Fig. 4. Fluorescence histograms of CMV-infected (A) and uninfected MRC-5 cells (B) stained with anti-pp65 monoclonal antibodies and screened by flow cytometer. Histogram (A) represents results of cells stained on day 5 post infection. The data for (A) and (B) were collected with the same instrument settings. LFL1, log of FITC fluorescence intensity for line 1. Line 1 respresents the area which was arbitrary set based on the negative control sample which represents the proportion, 82%, of pp65 containing MRC-5 cells.

For the sake of comparison, PMNL from 79 kidney transplant recipients were tested for the presence of CMV specific pp65 antigen by both AA and FCA. Samples tested included 35 AA-positive and 44 AA-negative kidney transplant recipients. As it is seen in Fig. 5 the AA can be evaluated microscopically to identify AA-negative and AA-positive cells. Fig. 6 shows the distribution of AA-positive samples by months after transplantation in relation to clinical presentation. As can be seen from the figure, fever was a common clinical complication among AA-positive kidney transplant recipients throughout the period of 6 months post transplantation. On the other hand, AA-positive kidney transplant recipients suffered from fever and leukopenia during the first four months post transplantation whereas systemic infections were seen only during the first and second month post transplantation. Since the level of immunosuppressive therapy (cyclosporin level) decreases with time after transplantation, it could contribute to the variations of clinical manifestations in AA-positive kidney transplant recipients. The following results were obtained by the AA: 12 patients had 5-20 AA positive cells/5×10^4, 11 patients 21-50, 5 patients 51-100, 4 patients 101-200 and 3 more than 200. Studing these samples by FCA showed (Table 8) that the FCA was negative in all of the AA negative patients (n=44). As an example Fig. 7 shows the fluorescence histograms of PMNL from a kidney transplant recipient who had 200 CMV AA-positive cells. Samples with 5-20 AA positive cells per 5×10^4 showed an 0.6% positivity by FCA. The mean percentage of positivity increases proportionally with the number of AA positive cells reaching 9.4% when the number of AA positive cells was greater than 200. By comparing the percentage of AA-positive cells with the percentage of FCA (Table 8), we can conclude that FCA has a higher sensitivity than that of the AA. This, however, could be due to nonspecific fluorescence which may play a part in the seemingly higher sensitivity of the FCA. To elucidate the role of nonspecific staining more samples need to be examined. Fig. 8 shows a comparison between AA and FCA. The regression square (Rsq)= 0.927 calculated from the results indicates that there



(A) (B)

Fig. 5. The result of (A) AA-negative case (B) AA-positive case of kidney transplant recipients. A positive result viewed as dark brown to red-brown nuclear or perinuclear staining within PMNL, while a negative result demonstrates no specific staining of PMNL upon examination of the duplicate wells under a light microscope at 200x-400x magnification. Hematoxylin was used as a counterstain. This is a selected field, normally positive cells are very rare.

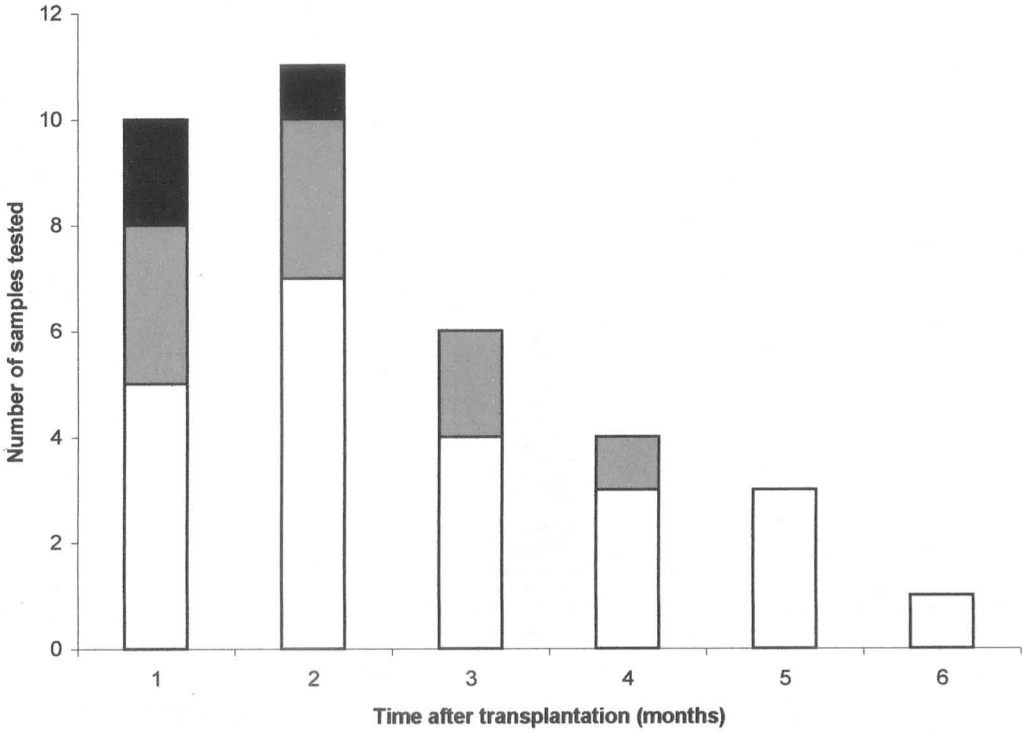
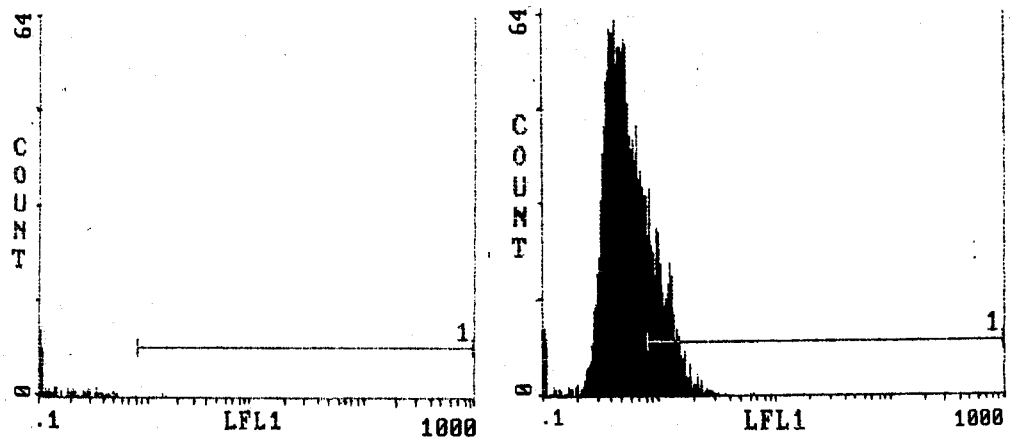


Figure 6. Distribution of AA-positive samples in months after transplantation with the clinical presentation. White bars represent patients with prolonged fever, gray bars represent patients with fever and leukopenia, black bars represent patients with systemic infection.



(A) Isotypically matched control.

(B) AA-positive patient.

Fig. 7. Fluorescence histograms of CMV-infected case from kidney transplant recipients (the Number of AA-positive PMNL >200 cells per 50,000) stained with (A) isotypically-matched control (B) anti CMV-monoclonal antibodies (IgG1). The data for (A) and (B) were collected with the same instrument settings. LFL1, log of FITC fluorescence intensity. The horizontal line represent the gate arbitrary set to estimate the number of positively stained cells (it excludes 98% of cells stained with the control antibody) which represent the proportion, 7.8%, of pp65-containing PMNL.

Table 8

Number of antigen-positive PMNL as determined by AA and FCA.

No of Patients tested	Mean No of AA positive cells[a] (%)	CMV-related Clinical Symptoms	FCA fluorescence %(±SD)	FCA Mean FI value[b]
44	2 (0.004)	no symptoms, AA negative	0.01 (±0.01)	0.4
12	12 (0.024)	fever	0.6 (±0.02)	0.8
11	34 (0.07)	fever	1.8 (±0.02)	3.6
5	74 (0.15)	fever, leukopenia,	2.6 (±0.04)	5.8
4	173 (3.5)	fever, leukopenia,	4.2 (±0.09)	8.7
3	370 (7.4)	fever, leukopenia, hepatitis and elevated liver enzymes	9.4 (±0.05)	11.8

a: AA is considered positive when 5 or more pp65 positive cells per 50,000 are detected.

b: Mean FI values were obtained by gating on the population of interest and calculating the mean fluorescence intensity.

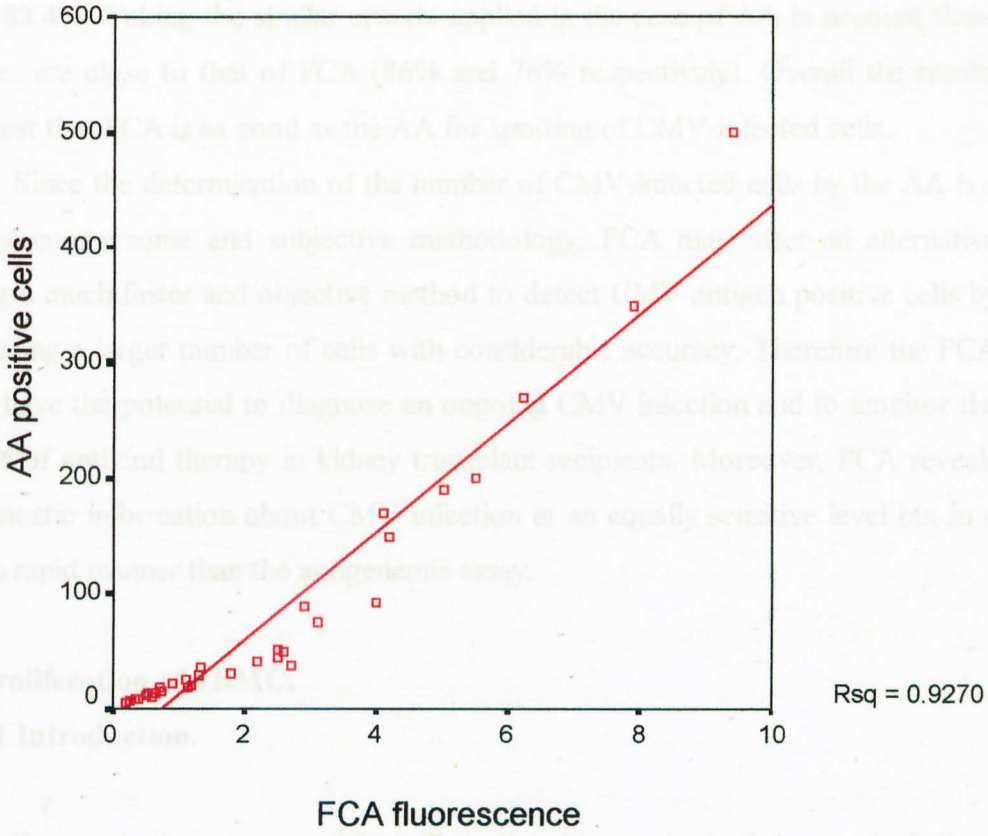


Fig. 8. Correlation between the fluorescence of CMV-infected PMNL screened by flow cytometry to detect the proportion of cells containing the pp65 antigen and the number of AA-positive PMNL containing the pp65 antigen in 35 kidney transplant recipients with active CMV infection.

is a strong correlation between AA and FCA ($p<0.0001$). It is important to know how FCA results correlates with the CMV-related clinical manifestations. FCA had a negative predictive value of 97.5% while its positive predictive value was 82.4%. Taking the similar criteria applied in the case of AA in account these values are close to that of FCA (86% and 76% respectively). Overall the results suggest that FCA is as good as the AA for spotting of CMV-infected cells.

Since the determination of the number of CMV-infected cells by the AA is a rather cumbersome and subjective methodology, FCA may offer an alternative being a much faster and objective method to detect CMV antigen positive cells by screening a larger number of cells with considerable accuracy. Therefore the FCA may have the potential to diagnose an ongoing CMV infection and to monitor the effect of antiviral therapy in kidney transplant recipients. Moreover, FCA reveals prognostic information about CMV infection at an equally sensitive level but in a more rapid manner than the antigenemia assay.

3.2 Proliferation of PBMC.

3.2.1 Introduction.

Due to the importance of the cell-mediated immunity in the control of CMV infection (section 1.5.2) the next objective was to study some aspects of the cellular immune responses of kidney transplant recipients with and without active CMV infection. In this respect PBMC responses to mitogen (PHA) and CMV antigen (CMV-infected MRC-5 cells) were investigated. PBMC were obtained from the 79 kidney transplant recipients by Ficoll-Hypaque density gradient centrifugation (section 2.9.1) and their stimulation indices (S.I.) to PHA and CMV-antigen were determined (section 2.9.2 and 2.9.3). The proliferation of PBMC to PHA and CMV antigens was carried out for each patient in triplicate along with a negative control to ensure validity of the data.

During active CMV infection cytokines may play an important role in determining the outcome of infection (section 1.6.3). Due to the strong influence of Th1- and Th2-type responses on the outcome of CMV infections, Th1- and

Th2- type cytokine profiles of kidney transplant recipients with and without active CMV infection were investigated. The levels of Th1-type cytokines (IL-2, TNF-α, IFN-γ) and Th2-type cytokines (IL-4, IL-10) were measured in the supernatant of PBMC stimulated either with PHA or with CMV antigen by ELISA (section 2.10). The determination of the levels of Th1- and Th2- type cytokines gave an opportunity to calculate the ratio of Th1- and Th2-type cytokines. It was presumed that the ratio of Th1- and Th2-type cytokines would give a better parmeter for characterising the association between CMV infection and cytokine production than measuring their levels alone.

3.2.2 PHA-stimulated cell mediated immune responses.

PHA induced-proliferation of PBMC from 35 AA-positive and 44 AA-negative kidney transplant recipients were assessed by thymidine uptake (section 2.9.2). Fig. 9 shows the stimulation indices of PBMC from AA-positive and AA-negative kidney transplant recipients after stimulation with PHA. Stimulation index of greater than 50 was considered to be a positive proliferative response. The stimulation indices were found at a significantly lower level in infected individuals as compared to uninfected individuals. The calculated p value showed a high level of difference between CMV-infected and non-infected patients. The results are demonstrated in Fig. 9. When the proliferative response of PBMC was related to the number of AA-positive cells, it was found that the stimulation indices in the AA-positive kidney recipients was inversely related to the degree of antigen positivity (Rsq=0.75, Fig. 10); in other words, patients who had a higher number of CMV-antigen positive cells showed lower stimulation indices than did patients who had lower number of CMV-antigen positive cells. The S.I. was as low as 2 when the number of CMV-pp65 positive cells was 500 while it was as high as 60 when the number of CMV-pp65 positive cells was around 10. The results are shown in Fig. 10.

Fig. 11 shows the correlation between the mean value of PHA S.I. for AA-positive kidney transplant recipients and the clinical events. As can be seen from

Fig. 11 the S.I. was lower in patients with severe, systemic CMV-related disease when compared to those with less severe clinical manifestations (fever, fever and leukopenia).

Table 9

Mitogen-induced PBMC proliferation* in AA-positive and AA-negative kidney transplant recipients.

	Proliferation positive	Proliferation negative	Total
AA-positive	**2**	**33**	**35**
AA-negative	**43**	**1**	**44**
Total	**45**	**34**	**79**

* PBMC proliferation was considered as positive when the S.I. was ≥50.

As can be seen from Table 9, of the 35 AA-positive patients 33 (94%) were proliferation-negative. On the other hand, of the 44 AA-negative patients only 1 (2%) was unresponsive to PHA. In the next phase of the study, PHA stimulation indices of PBMC from AA-positive kidney recipients were examined according to their CMV status; firstly when they were free of CMV infection (negative in the AA), secondly when they had an active CMV infection (presenting with symptoms and being positive in the AA), and thirdly when they recovered from the infection.

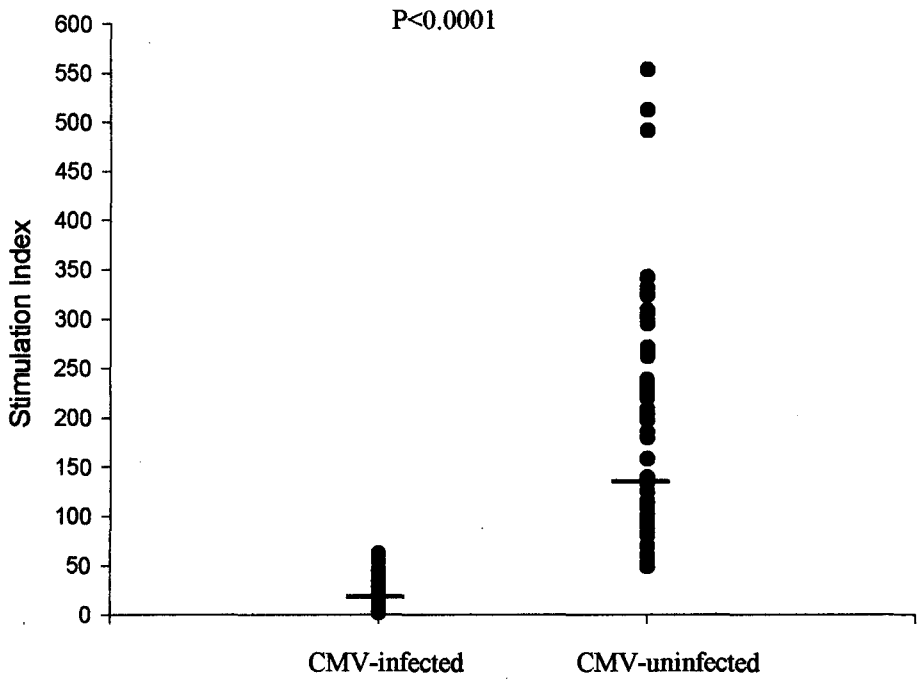


Figure 9. Stimulation indices (S.I.) of 35 AA-positive and 44 AA-negative kidney transplant recipients after stimulation of PBMC with PHA for 96 hours. Horizontal lines represent medians.

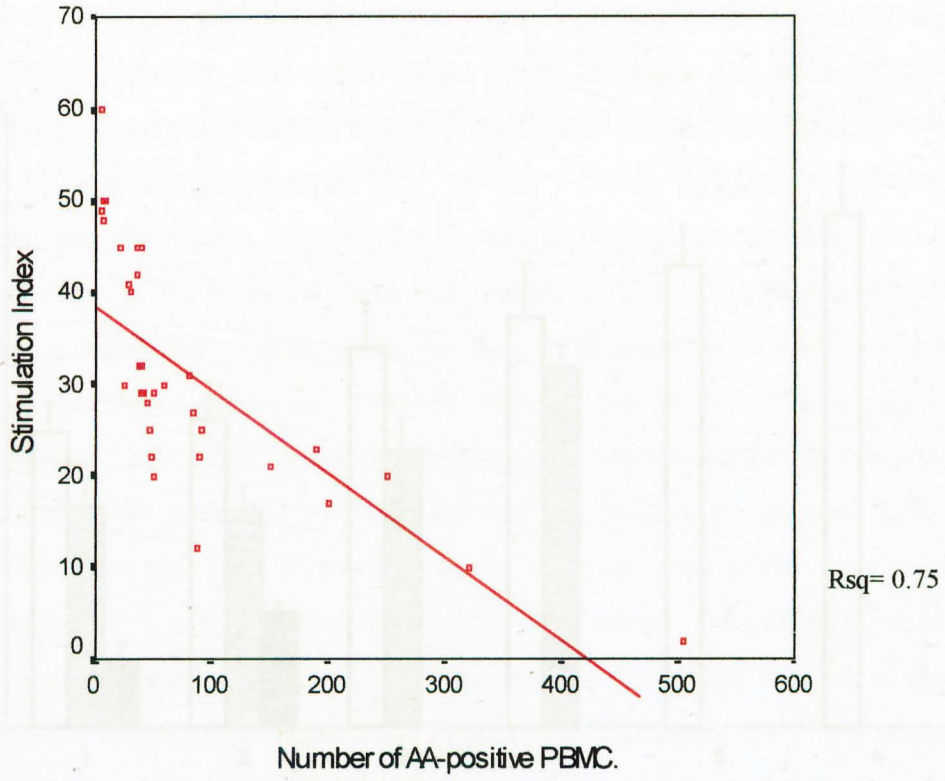


Fig. 10. Correlation between PBMC proliferation (stimulation index) in response to mitogenic stimulation and the number of AA-positive PMNL in kidney transplant recipients with active CMV infection.

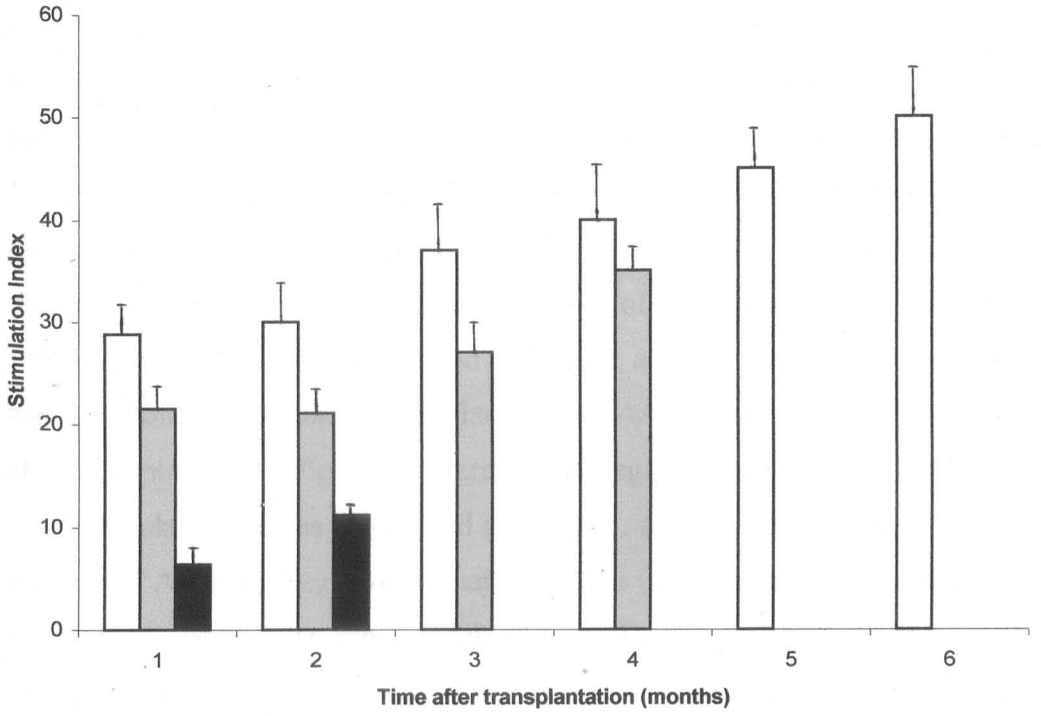


Figure 11. Correlation between the mean value of PHA stimulation indices for AA-positive kidney transplant recipients and the clinical events. White bars represent patients with fever, gray bars represent patients with fever and leukopenia, black bars represent patients systemic infections.

Data are presented in Fig. 12 As it is shown the indices were at a lower level during active CMV infection (B, median S.I.= 24, $p<0.0005$) when compared with the indices obtained before the active CMV infection (A, median S.I.=80, $p<0.0005$). The indices though marginally increased remained relatively low after the AA became negative again (C, median S.I.=28, $p<0.05$). A comparison between the stimulation indices of AA-negative, symptom-free recipients (Fig. 12D) and that of the AA-positive patients before active CMV infection (Fig. 12A) showed a significant difference in the S.I. ($p<0.0005$). This implies that the reduced immunoresponsivness may be one of the factors that triggers the activation of CMV. PHA stimulation indices for a selected number of AA-positive and symptomatic patients (n=5) who became AA-positive on the second month after transplantation (Fig. 13A) were in the negative range (<50). On the other hand, stimulation indices determined monthly for a period of 6 months for selected number of AA-negative, asymptomatic patients (n=5), though fluctuated, always remained in the positive range (>50) (Fig. 13B). The result of PHA stimulation clearly shows that active CMV infection influences certain cellular responses measured by stimulation indices. Results suggest that active CMV infection has a diminishing effect on the cellular response. Though stimulation indices are related to cellular response, this approach cannot give information about the subsets of cells involved in the interaction with active CMV infection. Therefore the study has been extended to analyse the levels of cytokines produced by different subsets of cells involved in the process of the immune response.

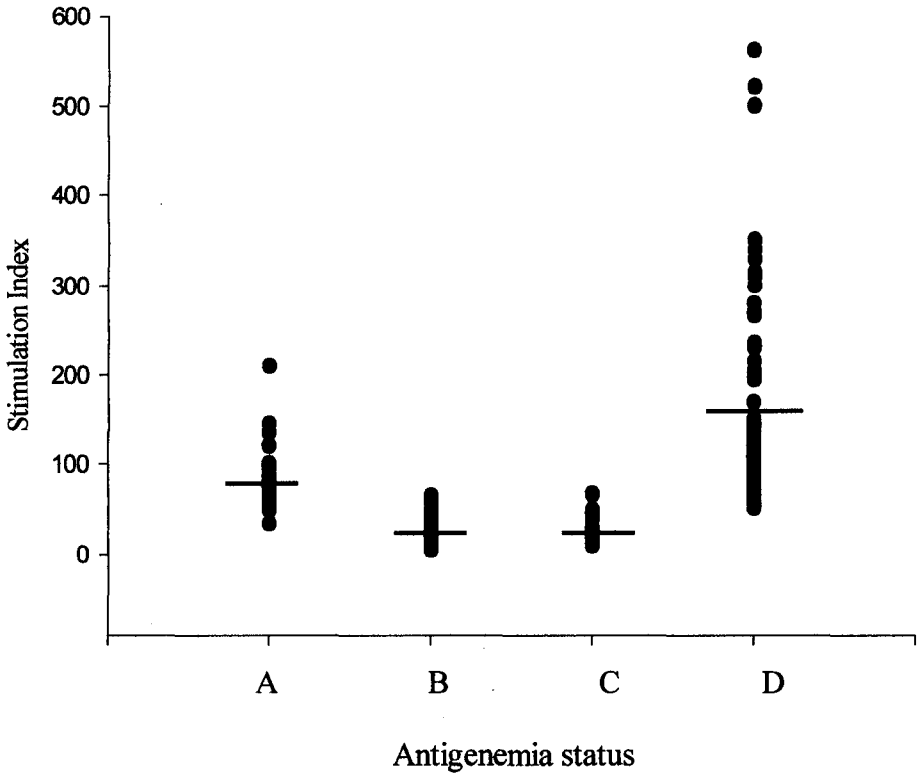


Fig. 12. Stimulation indices of PHA-stimulated PBMC from kidney transplant recipients when patients were AA-negative (A) when these patients became AA-positive, (B) and when the patients became AA-negative again (C). (D) stimulation indices of AA-negative patients. Horizontal lines represent medians.

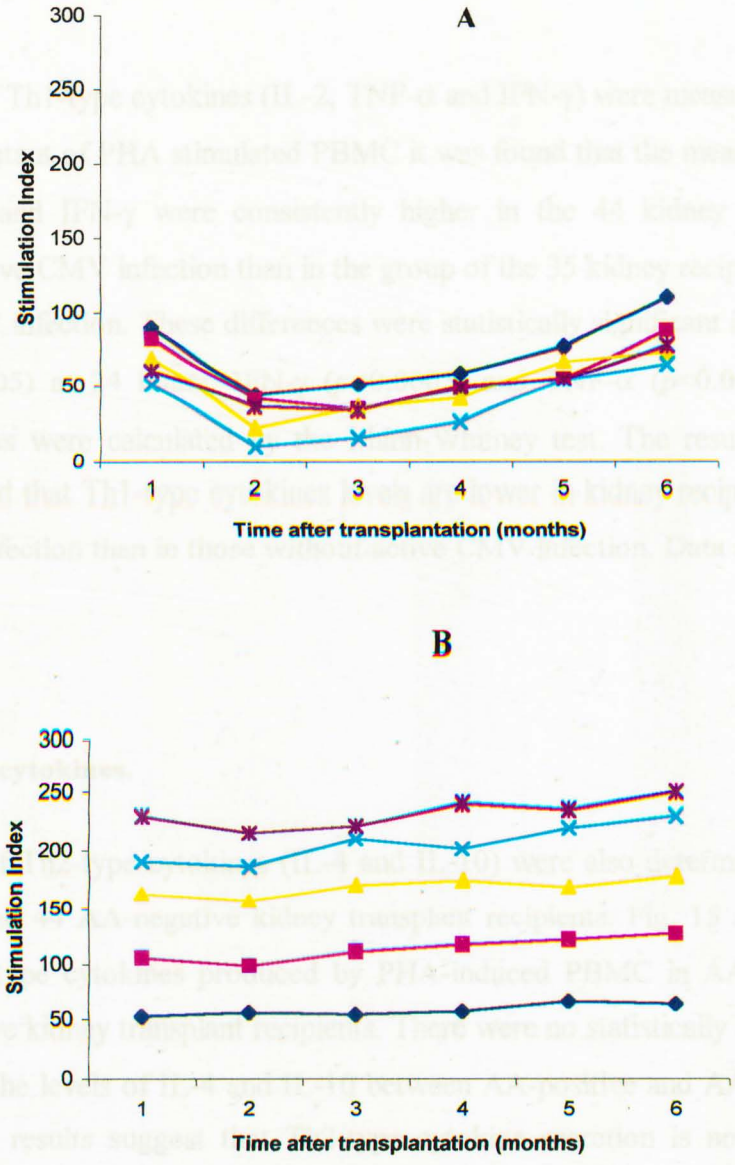


Figure 13. Response to PHA stimulus of successive samples obtained from (A) AA-positive and symptomatic patients (B) AA-negative and asymptomatic patients (selected data).

3.3 Th1-type cytokines.

When levels of Th1-type cytokines (IL-2, TNF-α and IFN-γ) were measured in the culture supernatant of PHA stimulated PBMC it was found that the mean levels of IL-2, TNF-α and IFN-γ were consistently higher in the 44 kidney recipients without an active CMV infection than in the group of the 35 kidney recipients with an active CMV infection. These differences were statistically significant in the case of IL-2 ($p<0.05$) at 24 hours, IFN-γ ($p<0.0005$) and TNF-α ($p<0.005$) at 96 hours. *P* values were calculated by the Mann-Whitney test. The results of this analysis showed that Th1-type cytokines levels are lower in kidney recipients with active CMV infection than in those without active CMV infection. Data are shown in Fig. 14.

3.4 Th2-type cytokines.

Levels of Th2-type cytokines (IL-4 and IL-10) were also determined in 35 AA-positive and 44 AA-negative kidney transplant recipients. Fig. 15 shows the levels of Th2-type cytokines produced by PHA-induced PBMC in AA-negative and AA-positive kidney transplant recipients. There were no statistically significant differences in the levels of IL-4 and IL-10 between AA-positive and AA-negative groups. These results suggest that Th2-type cytokine secretion is not affected significantly by active CMV infection. In order to get a better picture on the production of cytokines of kidney transplant recipients with and without active CMV infection the ratio of Th1/Th2 cytokines was investigated.

3.5 Th1/Th2 cytokines ratios produced by PHA-stimulated PBMC.

The ratio of Th1 to Th2-type cytokines in a given sample is considered to be of greater significance than the levels of cytokines alone. It seems to give a better understanding of the association between CMV infection and cytokine production. Therefore, the ratios of different Th1 to Th2-type cytokines produced by PHA-

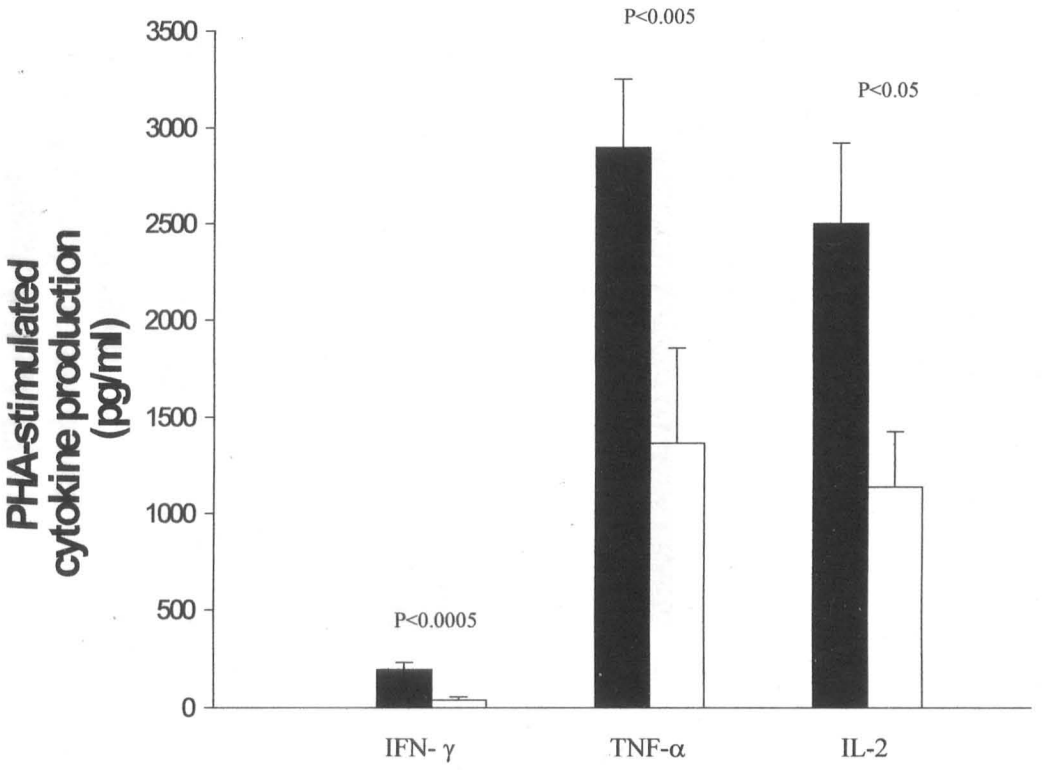


Fig. 14. Levels of Th1-type cytokines produced by PHA-induced PBMC after 24 hours of culture for IL-2 and 96 hours of culture for IFN-γ and TNF-α as measured by ELISA. Dark bars represent mean levels (±SEM) of the cytokines in AA-negative individuals; white bars represent mean levels in AA-positive subjects.

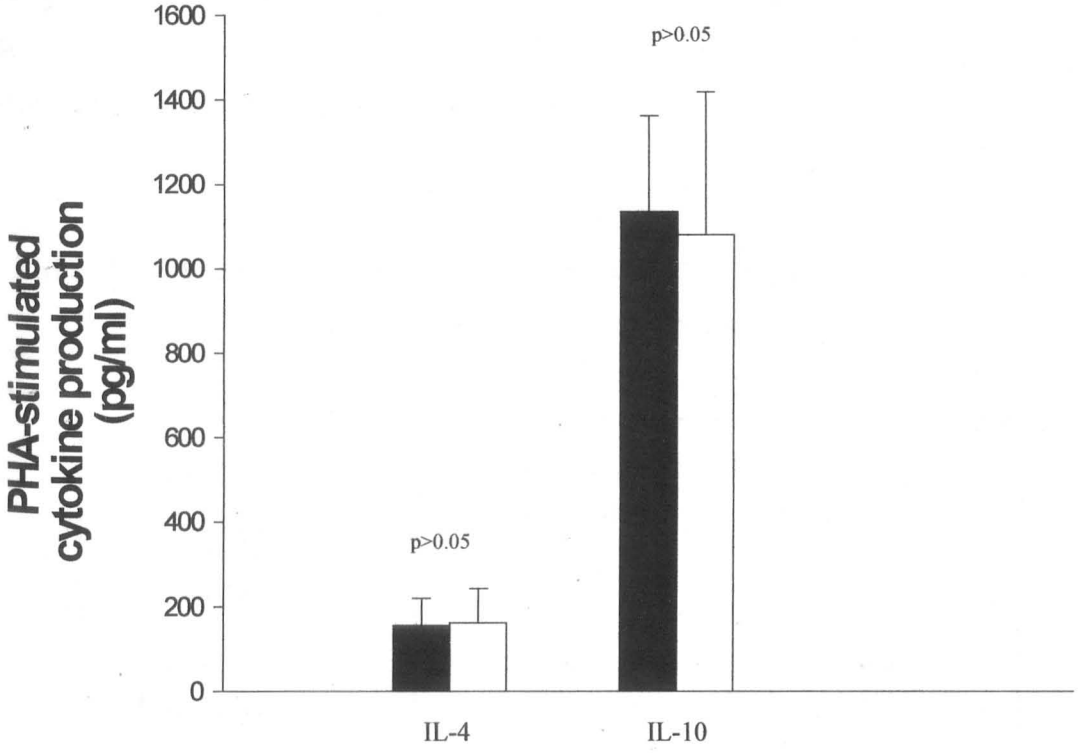


Fig. 15. Levels of Th2-type cytokines produced in the supernatant of PHA-induced PBMC after 96 hours of culture as measured by ELISA. Dark bars represent mean levels (±SEM) of the cytokines in AA-negative individuals; white bars represent mean levels in AA-positive subjects.

stimulated PBMC were calculated. The mean values of Th1-type cytokines were compared to that of Th2-type cytokines produced by PBMC of kidney transplant recipients with and without active CMV infections. The difference between the two groups in some of the Th1:Th2 ratios was found to be striking. Table 10 depicts the ratios of Th1 to Th2-type cytokines.

Table 10

Th1: Th2 cytokines ratios of PHA-stimulated PBMC from AA-positive and AA-negative kidney transplant recipients.

Th1:Th2 Ratio	AA-negative patients	AA-positive patients
After 96 hours of culture		
TNF-α:IL-4	18.6	8.4
TNF-α:IL-10	2.6	1.3
IFN-γ:IL-4	1.3	0.2
IFN-γ:IL-10	0.18	0.04

As can be seen from Table 10, the TNF-α:IL-4 ratio at 96 hours were lower in the AA-positive group as compared to the AA-negative group, indicating a lower Th1-bias in the AA-positive group. In the cases of TNF-α:IL-10, IFN-γ: IL-4 and IFN-γ:IL-10 the differences in ratios are not as striking, even though the ratios are consistently lower in infected individuals. Thus, the results support the view that there is a general trend of a lower production of Th1-type cytokines by the AA-positive group than by the AA-negative group. Studing the responses of PHA-stimulated PBMC of kidney transplant recipients provided data about a decreased cellular immune response of CMV infected individuals. Exploring this problem

further, responses of PBMC to CMV antigen (CMV-infected MRC-5 cells) were investigated.

3.6 CMV infected fibroblast-stimulated cell mediated immune responses.

The previous section described the responses of PBMC stimulated with a mitogen which provides a non-specific stimulus. This section describes the stimulation of PBMC with an antigenic stimulus, i.e. CMV antigens. This line of experimentation is expected to provide information on the specific response of PBMC to CMV antigens. PBMC from kidney transplant recipients were stimulated with CMV-infected MRC-5 cells (section 2.9.2) and the induced-proliferation was assessed by measuring the thymidine uptake (section 2.9.3). S.I. of >3 was considered to be a positive response.

Table 11

PBMC proliferation in response to CMV antigens in AA-positive and AA-negative kidney transplant recipients.

	Proliferation positive (%)	Proliferation negative (%)	Total
AA-positive	4(11%)	31(89%)	35
AA-negative	40(91%)	4(9%)	44
Total	44	35	79

As can be seen from Table 11, of the 35 AA-positive patients 31 (88%) were proliferation-negative; while only 4 (9%) of the 44 AA-negative patients were unresponsive to CMV antigens. PBMC of CMV-infected transplantees responded poorly to stimulation by CMV antigens as compared to CMV-negative transplantees. Fig. 16 depicts the stimulation indices of PBMC obtained from 35 AA-positive and 44 AA-negative kidney transplant recipients after stimulation with

CMV. The PBMC of AA-positive kidney transplant recipients showed significantly lowered stimulation indices in response to stimulation with CMV-infected fibroblasts than did AA-negative kidney transplant recipients. Median levels of stimulation indices were significantly lower in AA-positive kidney transplant recipients (2.0) as compared to AA-negative kidney transplant recipients (10.5) with a p value of <0.0001. In the group of AA-positive recipients the number of CMV-pp65 positive cells varied between 10-500; the S.I. reached as low as 1 when the number of CMV-pp65 positive cells was 500 and as high as 11.5 when the number of CMV-pp65 positive cells was around 10.

Fig. 17 shows the stimulation indices of PBMC from AA-positive before (A), during (B) and after (C) recovery from active CMV infection (being free of antigen positive cells and symptoms). As can be seen from Fig. 17, the stimulation indices were decreased during active CMV infection (median S.I=2, $p<0.005$) as compared with the indices of the same group of kidney transplant recipients before active CMV infection (median S.I.= 5.7, $p<0.005$). On the other hand, the indices of the same group of kidney transplant recipients after active CMV infection (C) increased marginally (median S.I.= 2.8, $p= 0.08$). When comparing the stimulation indices of AA-negative and symptom-free recipients (Fig. 17D) with that of the AA-positive patients before active CMV infection occurred (Fig. 17A) a significant difference in the S.I. ($p<0.002$) could be seen. This implies that reduced immunoresponsivness may be one of the factors that triggers the activation of CMV. Stimulation indices by CMV antigen for a selected number of AA-negative, asymptomatic patients (n=5) were followed monthly for a period of 6 months. Though there were some changes in the levels of the S.I., it remained in the positive range (>3) during the study period(Fig. 18A). On the other hand, when lymphocytes of a selected AA-positive and symptomatic patients (n=5) who became AA-positive on the second month after transplantation were stimulated by the CMV antigen the S.I. values remained in the negative range (<3) (Fig. 18B). As it is shown, S.I. values for these patients started to increase when the CMV infection was over. Therefore, these results suggest that an active CMV infection

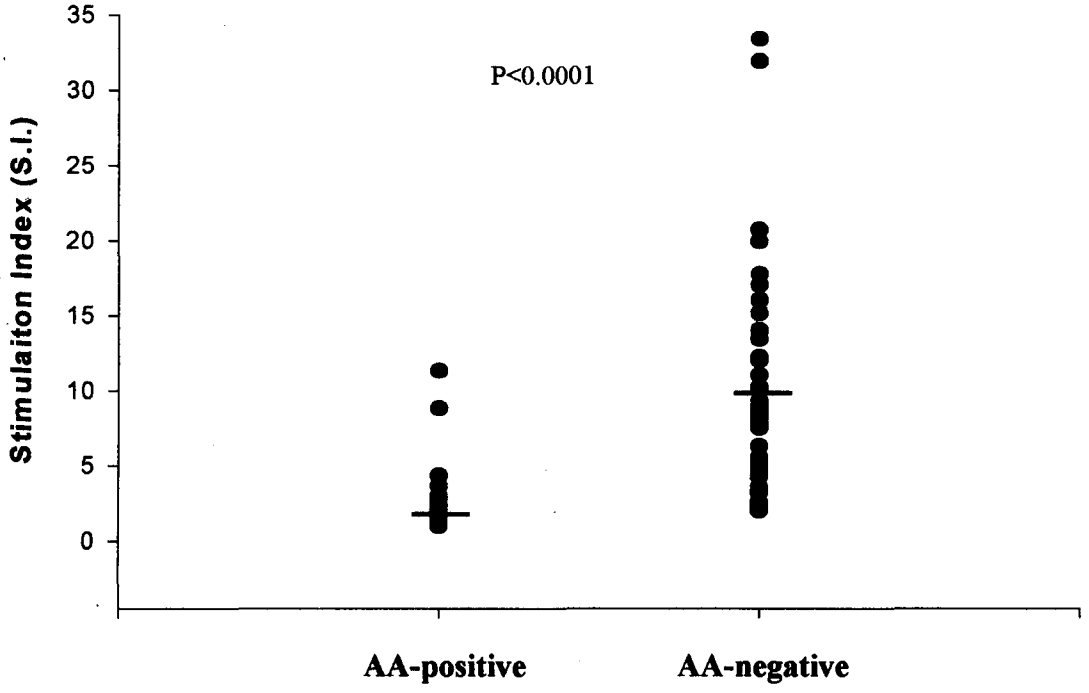


Fig. 16. Stimulation indices of PBMC from 35 AA-positive and 44 AA-negative kidney transplant recipients in response to stimulation with CMV-antigens (CMV-infected MRC-5) for 96 hours. Horizontal lines represent medians.

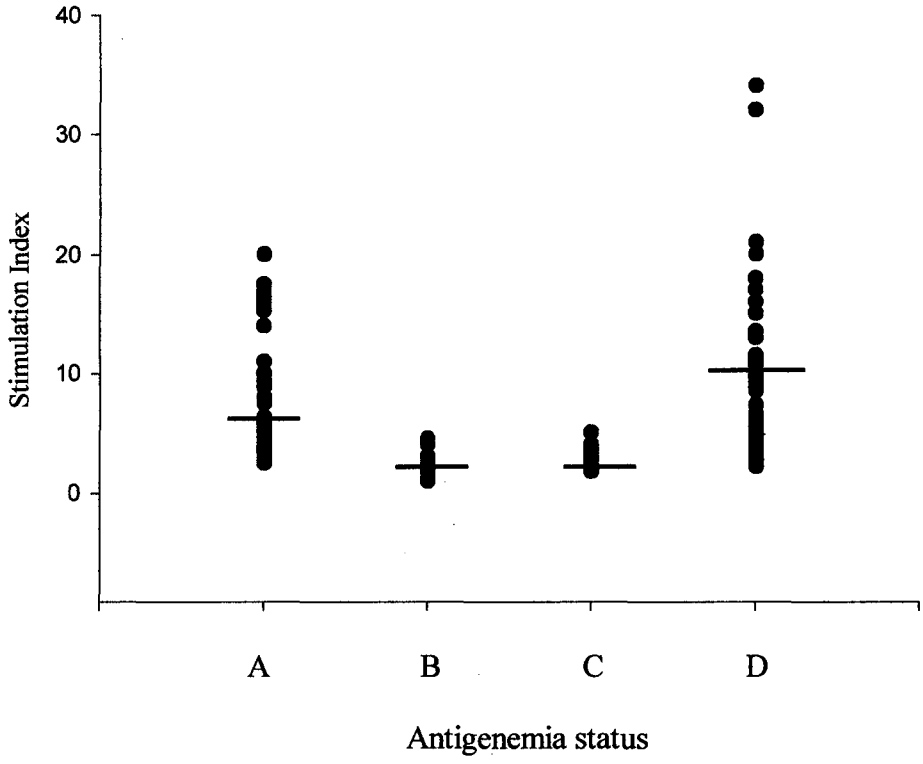
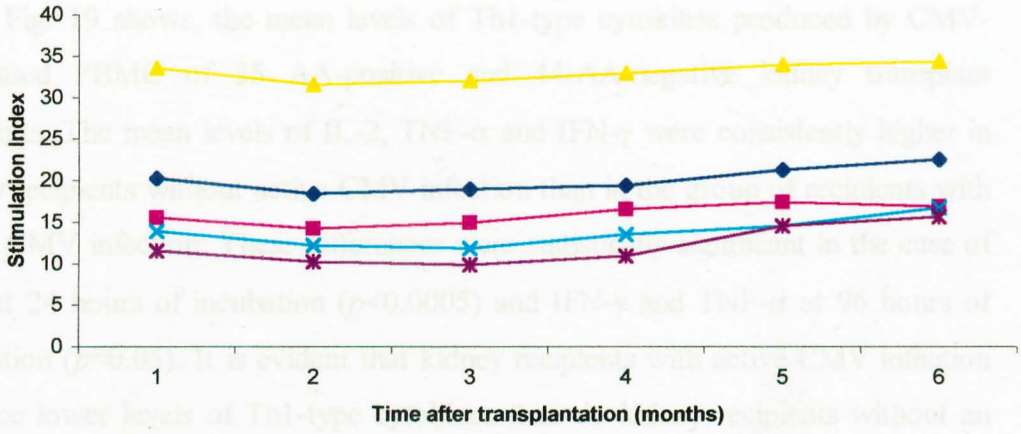


Fig. 17. Stimulation indices of CMV-stimulated PBMC of kidney transplant recipients when patients were AA-negative (A) when these patients became AA-positive, (B) and when the patients became AA-negative again (C). (D) stimulation indices of AA-negative patients. Horizontal lines represent medians.

(A)



(B)

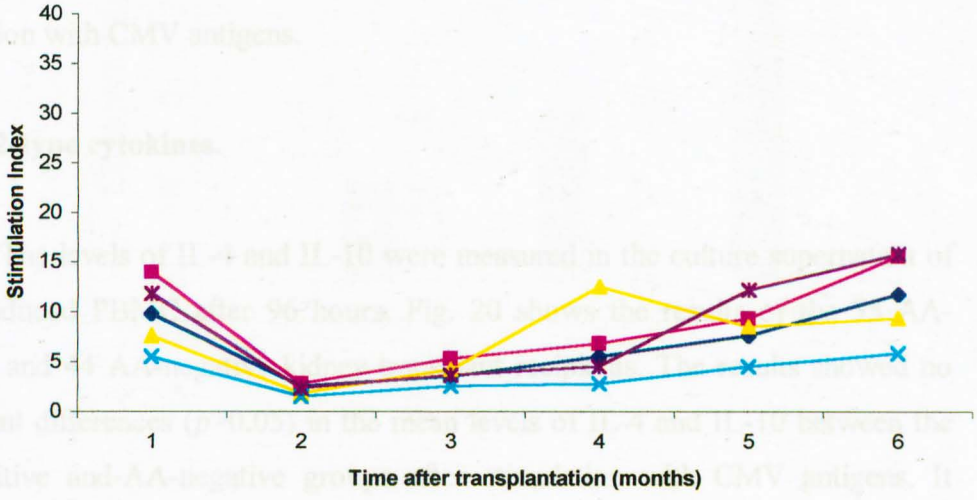


Figure 18. Stimlation indices from (A) five AA-negative kidney transplant recipients, (B) from five AA-positive kidney transplant recipients stimulated with CMV-infected MRC-5 over a period of six months.

may have a suppressive effect on the proliferative response of PBMC by CMV antigen.

3.7 Th1-type cytokines.

Fig. 19 shows, the mean levels of Th1-type cytokines produced by CMV-stimulated PBMC of 35 AA-positive and 44-AA-negative kidney transplant recipients. The mean levels of IL-2, TNF-α and IFN-γ were consistently higher in kidney recipients without active CMV infection than in the group of recipients with active CMV infection. These differences were statistically significant in the case of IL-2 at 24 hours of incubation ($p<0.0005$) and IFN-γ and TNF-α at 96 hours of incubation ($p<0.05$). It is evident that kidney recipients with active CMV infection produce lower levels of Th1-type cytokines than do kidney recipients without an active CMV infection. Therefore, these data suggest that active CMV infection is associated with a decrease in the production of Th1-type cytokines produced after stimulation with CMV antigens.

3.8 Th2-type cytokines.

The levels of IL-4 and IL-10 were measured in the culture supernatant of CMV-induced PBMC after 96 hours. Fig. 20 shows the results of the 35 AA-positive and 44 AA-negative kidney transplant recipients. The results showed no significant differences ($p>0.05$) in the mean levels of IL-4 and IL-10 between the AA-positive and-AA-negative groups after stimulation with CMV antigens. It appears that Th2-type cytokines production are not influenced significantly by active CMV infection in kidney transplant recipients.

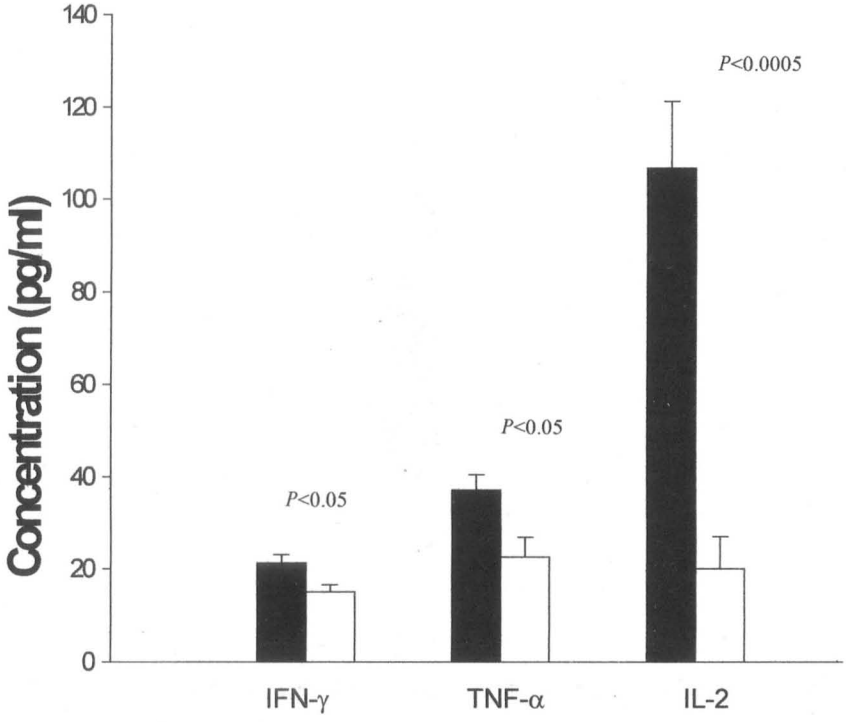


Fig. 19. Mean levels of Th1-type cytokines produced after stimulation of PBMC with CMV-infected MRC-5 cells. Levels of IL-2 were measured at 24 hours and levels of IFN-γ, TNF-α at 96 hours. Dark bars represent mean level (±SEM) of the cytokines in AA-negative individuals; white bars represent mean levels in AA-positive kidney transplant recipients.

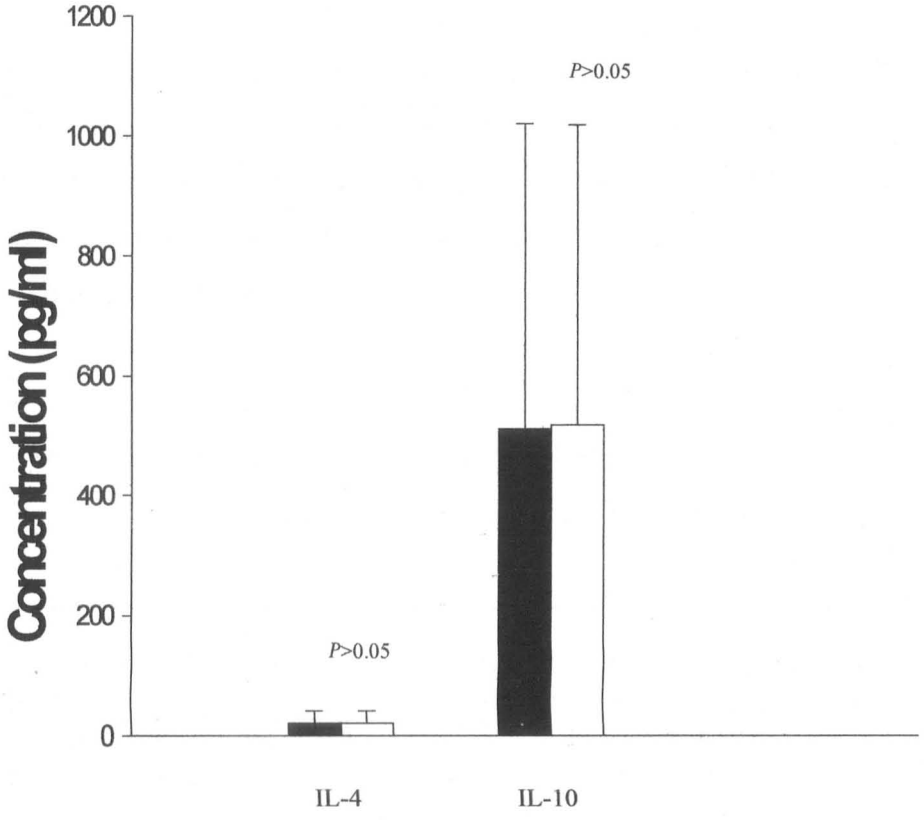


Figure 20. Mean levels of Th2-type cytokines produced after stimulation of PBMC with CMV-infected MRC-5 cells. Levels of IL-4 and IL-10 were measured at 96 hours. Dark bars represent mean levels (±SEM) of the cytokines in AA-negative kidney transplant recipients; white bars represent mean levels in AA-positive kidney transplant recipients.

3.9 Th1/Th2 cytokines ratios produced by CMV-stimulated PBMC.

The ratios of different Th1 to Th2-type cytokines produced by CMV-stimulated PBMC were calculated. The mean values of Th1-type cytokines were compared to that of Th2-type cytokines produced by PBMC of kidney transplant recipients with and without active CMV infections. Table 12 depicts the ratios of Th1 to Th2-type cytokines.

Table 12

Th1: Th2 cytokines ratios of CMV-stimulated PBMC of AA-positive and AA-negative kidney transplant recipients.

Th1:Th2 Ratio	**AA-negative patients**	**AA-positive patients**
After 96 hours of culture		
TNF-α:IL-4	2	1
TNF-α:IL-10	0.08	0.04
IFN-γ:IL-4	1.1	0.8
IFN-γ:IL-10	0.04	0.03

As can be seen from Table 12, the TNF-α:IL-4 and TNF-α:IL-10 ratio at 96 hours were lower in the AA-positive group as compared to the AA-negative group, indicating a lower Th1-bias in the AA-positive group. In the cases of IFN-γ: IL-4 and IFN-γ:IL-10 the differences in ratios are not as striking, even though the ratios are consistently lower in AA-positive kidney transplant recipients. Thus, data show that there is a general trend of a lower production of Th1-type cytokines by the AA-positive group than by the AA-negative group.

3.10 Immunophenotyping analysis of cells in the peripheral blood of AA-positive and AA-negative kidney transplant recipients.

It is known that CMV infection causes changes in the counts of lymphocyte subsets in peripheral blood (Beik *et al.*, 1998; Belles-Isles *et al.*, 1998). It is of great importance to enumerate percentage of different phenotype subsets during active CMV infection for diagnostic and prognostic purposes. Whole blood from AA-positive and negative kidney transplant recipients were analyzed by flow cytometry to detect changes, if any, in the number of different cellular subsets. Each patient sample was stained by antibodies specific to $CD2^+$ (pan T), $CD3^+$ (mature T), $CD4^+$ (T helper), $CD8^+$ (T suppressor), $CD26^+$ (T activated), $CD16^+$/ $CD56^+$ (NK cell), $CD19^+$ (pan B) and $CD15^+$ (granulocytes) (section 2.12.1). For each monoclonal antibody an appropriate isotypically matched control was used in each run.

Fig. 21 shows the results of the mean percentage of cells for different lymphocyte phenotype subsets in peripheral blood of kidney transplant recipients with and without active CMV infection. The percentage of $CD3^+$ immunocompetent T lymphocytes and $CD4^+$ T lymphocytes were consistently higher in kidney recipients without an active CMV infection (AA-negative) than in the group of recipients with an active CMV infection (AA-positive). These differences were statistically significant in the case of $CD3^+$ ($p<0.05$) and $CD4^+$ ($p<0.005$). On the other hand between AA-positive and AA-nagative cases, in the percentage of cells expressing $CD2^+$, $CD8^+$, $CD16^+ + CD56^+$, $CD19^+$, $CD15^+$ there was no statistically significant diffference ($p>0.05$). Fig. 22 and 23 show the percentages of cells expressing $CD4^+$ and $CD3^+$ respectively in kidney transplant recipients before the detection of active CMV infection (A), during active CMV infection (B) and after the recovery from the CMV infection (C). It was found that there was a significant decrease in the percentage of both $CD4^+$ and $CD3^+$ positive cells in kidney recipients during active CMV infection ($CD4^+$ median = 22, $p<0.005$; $CD3^+$ median = 41 $p<0.05$) when compared to the percentage of the

same group of kidney recipients before active CMV infection ($CD4^+$ median = 36, $p<0.005$; $CD3^+$ median = 59, $p<0.05$) . After the loss of antigenemia the percentage of $CD4^+$ and $CD3^+$ cells in these patients were increased ($p<0.05$). It is evident that kidney recipients show a significant alteration in the number of $CD4^+$ and $CD3^+$ T cells according to their CMV antigenemia status. Therefore, these data suggest that CMV infection is associated with a decrease in the number of $CD3^+$, $CD4^+$ T cells.

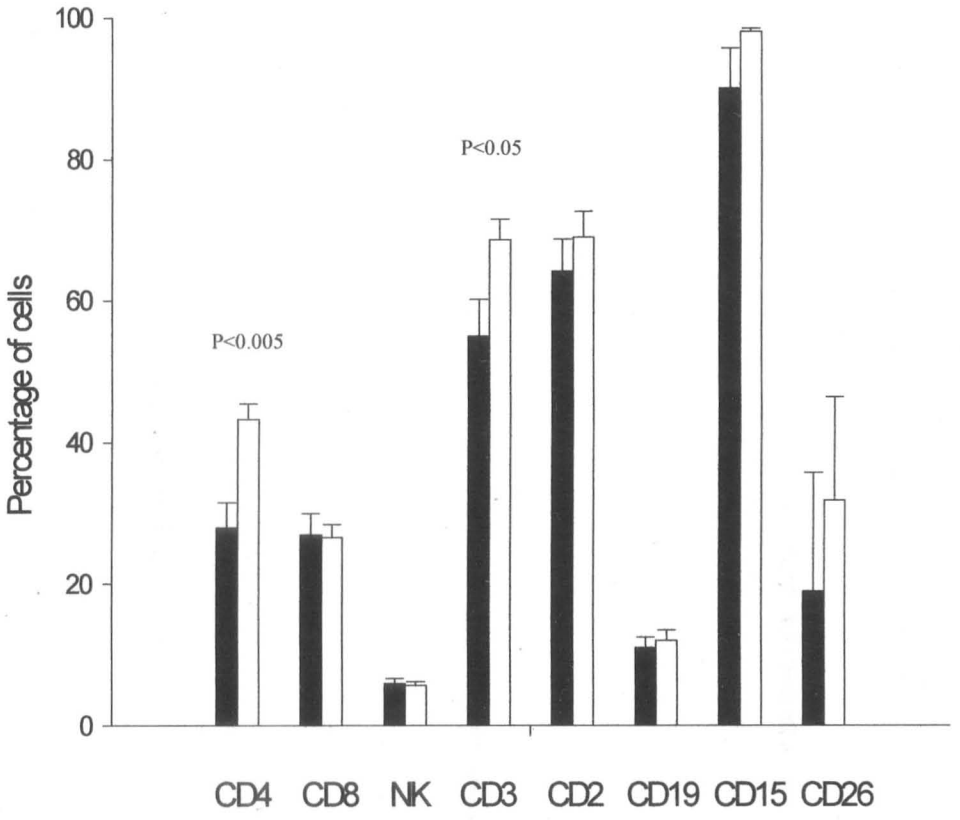


Figure 21. Immunophenotyping of cells in the peripheral blood of kidney transplant recipients with active CMV infection (35 patients) and without active CMV infection (44 patients) as detected by the AA. Dark bars represent mean percentage (±SEM) of the cellular antigens in AA-positive individuals; white bars represent mean percentages in AA-negative kidney transplant recipients.

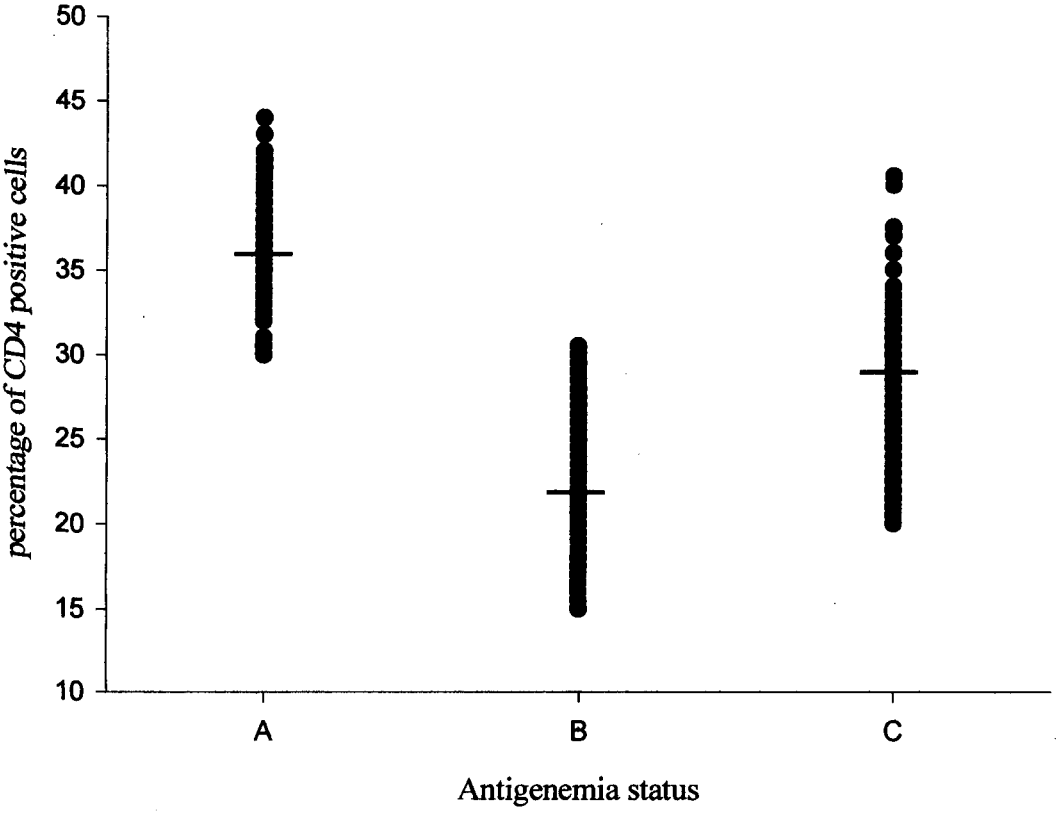


Figure 22. Percentages of $CD4^+$ cells as detected by flow cytometry in kidney transplant recipients before active cytomegalovirus infection (A) during active CMV infection, (B) and after active CMV infection (C) as determined by the antigenemia assay. Horizontal lines represent medians.

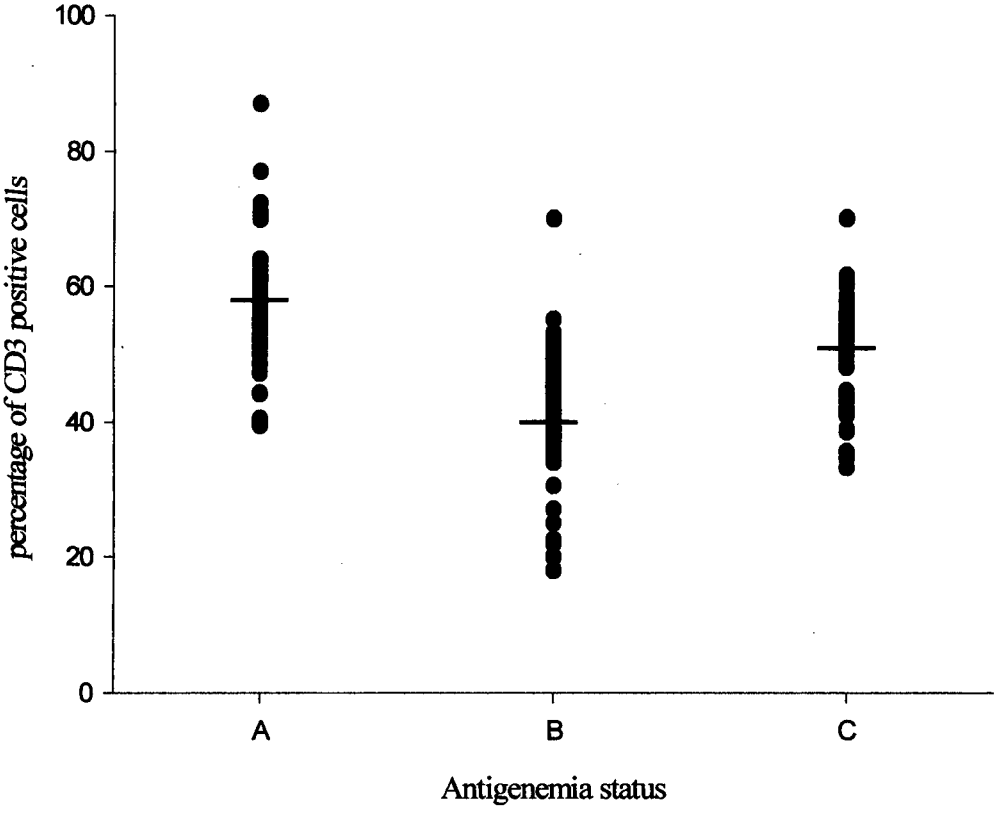


Figure 23. Percentages of $CD3^+$ cells as detected by flow cytometry in kidney transplant recipients before active cytomegalovirus infection (A) during active CMV infection, (B) and after active CMV infection (C) as determined by antigenemia assay. Horizontal lines represent medians.

4. DISCUSSION

CMV is a ubiquitous virus in the human population, and primary infections are mostly without clinical symptoms in healthy individuals. After primary infections the virus is never completely eliminated from the host but remains sequestered in a cell population from which it can reactivate. During latency there is a constant interaction between the virus and the immune system: the latent virus evades immune destruction most probably by inducing substances which block immune recognition and down-regulate immune responses. This is signified by the fact that in the event of local reactivation of CMV, viral proteins are promptly recognized and further multiplication of the virus is controlled by the immune response. Thus, in healthy individuals localized viral expression stimulates adequate immune response to prevent further spread of the virus. However, in immunocompromised individuals the balance between viral activity and immune responses shifts in favour of the virus and consequently severe CMV infections may occur. CMV continues to be an important post-transplant pathogen in organ transplant recipients and is responsible for a substantial fraction of the morbidity and mortality that follows organ transplantation (Hibberd and Snydman, 1995). Because of the growing number of allograft transplantations, this agent constitutes a major pathogen in hospitalized patients. It has been reported that between 60% and 100% of renal transplant recipients develop CMV infection (as measured by virus excretion) (Pollard, 1988).

Various antiviral agents have been used in attempts to treat established CMV disease (Alford, 1984). The preparations have included leukocyte interferon, interferon-stimulators (measles virus), transfer factor and nucleoside drugs, [iododeoxyuridine, fluorodeoxyuridine, cytosine arabinoside, adenine arabinoside (vidarabine), and acyclovir] (Alford, 1984). None of these regimens proved to be clinically useful. Recently, a derivative of acyclovir, 9-(1,3-dihydroxy-2-propoxymethyl) guanine (gancyclovir) and a previously known inhibitor of viral DNA polymerases, phosphonoformic acid (foscarnet), have been shown to have potent activity against CMV *in vitro*. The mechanism of action of gancyclovir is

thought to reside in its nucleic acid chain-terminating activity, while foscarnet appears to inhibit the activity of the viral DNA polymerase directly (Snoeck *et al.*, 1993). In post-transplant patients, initiation of therapy as early as possible during active CMV infection is important in the management of CMV infection. Therefore, early diagnosis and treatment of active CMV infection plays as important role in the control and management of this virus (Noble and Faulds, 1998).

Traditionally, CMV infection has been demonstrated by virus isolation and identification in cell culture or by the finding of inclusion bodies in biopsy specimens (Griffiths, 1984). Serologic testing has been used to confirm exposure to CMV but requires acute and convalescent sera demonstrating a fourfold rise in titer and is less reliable since immunosuppressed patients may not have a change in antibody titer in the presence of active CMV infection (Drew *et al.*, 1985). Although the combination of viral replication in tissue culture, a rising antibody titer, and inclusion bodies on histopathology is a sensitive and accurate indicator of virus infection, as long as 8 weeks may be required for virus indentification and confirmation of diagnosis (Griffiths, 1984). With the advent of effective antiviral chemotherapy for human CMV infections, more rapid and sensitive techniques are required to identify CMV infections during the acute stage of the illness (Boeckh *et al.*, 1996; Ljungman *et al.*, 1992; Snoeck *et al.*, 1993). The availability of antigenemia assay (AA), a method that detects the presence of CMV-specific antigen by monoclonal antibodies expressed mainly during active infection has been used to identify CMV infection directly in clinical specimens (Boeckh *et al.*, 1996, Griffiths *et al.*, 1984, van der Bij 1988a,b). AA can also be used as a quantitative marker of virus load that permits a detailed view of the interaction between CMV and the host. The advantage of this AA is that an identification of virus infection can be made in a number of hours only during active viral replication, it has an advantage over the highly sensitive PCR method that detects the viral genome regardless the activity of the virus. This technique, however, can be labor intensive and may not be useful for screening the large number of samples received in a clinical virology laboratory.

Flow cytometry is a technique which can be used to analyze a large number of specifically labeled cells and quantify their fluorescence. This technology may allow more rapid and sensitive detection of CMV-specific antigen and may be useful for the identification and quantification of CMV-infected cells in clinical samples. However, prior to utilization for diagnostic purposes, it must be standardized and compared to established methods. The parameters of FCA can be tested on human diploid fibroblast cells infected with CMV to establish the validity of such an approach. The results showed that FCA performed well in the detection of MRC-5 cells infected with CMV since it was possible to detect the presence of antigen presenting cells as early as one day post infection (section 3.1.2). When cells were stained with FITC-labeled monoclonal antibodies specific for the CMV-early matrix protein (pp65), the antigen-containing cells were detected by the FCA. Analysis of the sensitivity of FCA was investigated by testing for the presence of CMV pp65 antigen in PMNL of kidney transplant recipients. In these samples the FCA detected the pp65 antigen even when the number of AA positive cells was between 5-20 indicating that the FCA was at least as sensitive as the AA (section 3.1.3).

These results showed that the FCA detects the CMV pp65 antigen in PMNL of kidney recipients with active CMV infection. An advantage of the FCA is that large numbers of cells and samples can be screened in a short time with accuracy and efficiency. FCA has a potential to be used for the diagnosis of an ongoing CMV infections, to monitor the effect of antiviral therapy in kidney transplant patients and to analyze the mechanism of CMV infection. Since the FCA is based on the detection of an antigen, which appears in the leukocytes only during active viral replication, it also has an advantage over the highly sensitive PCR method that detects the viral genome regardless the activity of the virus. Further studies should be carried out to study the possibility of using the technique of immunofluorescent staining and flow cytometry on direct clinical samples and it maybe able to provide results in less time without the need to purify PMNL from peripheral blood samples. This method may also be useful for the detection of other pathogens, where specific monoclonal antibodies are available.

Due to the monocytotropic character of CMV, it exhibits an intimate relationship with the host immune system, and is therefore of great interest to study this relationship and to understand the nature of immune responses against CMV infection in transplant patients. The host immune response to CMV involves specific as well as nonspecific immune surveillance mechanisms, which undoubtedly play roles in clearing virus infection. This is a complex process in general and particularly complex in immunocompromised patients because CMV itself induces immunosuppression and can interact with cells of the immune system (Koszinowski *et al.*, 1990). One of the most marked effect of CMV-infected monocytes is their immunosuppressive effects on lymphocyte proliferative responses. During acute CMV infection lymphocytes from mononucleosis patients do not respond to stimulation with the mitogen concanavalin A (con A) (Carney and Hirsch, 1981). When monocytes from seronegative donors are infected *in vitro* with CMV and added to autologous lymphocytes, there is no lymphoproliferation following con A stimulation (Carney and Hirsch, 1981). Similarly, *in vitro* infection of peripheral blood lymphocytes inhibits lymphoproliferative responses to polyclonal mitogen stimulation (Wahren *et al.*, 1986). For the prevention of an active CMV infection and disease, cellular immunity in general, and the action of cytotoxic T cells to CMV-infected target cells in particular, is the most important factor in the host defense mechanisms (Quinnan *et al.*, 1982; Reusser *et al.*, 1991; Riddel and Greenberg, 1994; Riddell *et al.*, 1992; Rook *et al.*, 1984). In kidney transplant recipients the outcome of CMV infection depends to a great extent on the activity of T cells (Mosmann and Coffman, 1989; Quinnan *et al.*, 1982 Reusser *et al.*, 1991; Rook *et al.*, 1984). PBMC proliferation to PHA activation was measured in this study as an indicator of the T cell responses in kidney transplant recipients with and without active CMV infections. Transplant recipients were grouped on the basis of being positive or negative in the AA. Lymphocytes were obtained from all patients and stimulated with PHA (section 2.9.2).

The results of PHA-induced proliferative responses of PBMC revealed that transplant recipients with an active CMV infection had lower levels of proliferation (S.I.<50) when compared to recipients without active CMV infections (S.I.≥50)

(section 3.3.2). Kidney transplant patients received an immunosuppressive drugs shortly after transplantation. Since patients became AA-positive between 1 and 3 months after transplantation, it is unlikely that cyclosporin A had an influence on the proliferation of PBMC or the levels of Th1-type cytokines. If cyclosporin played a role it would have an effect on AA-negative kidney transplant recipients. The reduction in the PHA-induced response of PBMC shows an inverse correlation with the number of pp65 antigen positive cells (Fig. 9). PBMC of recipients with high numbers (500/50,000) of pp65 antigen-bearing cells showed very low PHA-induced responses (S.I.<5). This may reflect the fact that an ongoing, active CMV infection has a diminishing effect on T cell response. This illustrates the importance of helper T cell responses, and is consistent with the well-recognized role of CMV-specific cytotoxic T cells in recovery from opportunistic CMV infections (Quinnan *et al.*, 1982; Reusser *et al.*, 1991; Riddell and Greenberg, 1994; Riddell *et al.*, 1992). Similar observations have been reported in relation to kidney transplant recipients; subjects with active CMV infection had diminished cytotoxic T cell responses (Rook *et al.*, 1984). This current study provides evidence that the decline in T cell response may be due to the immunosuppressive effect of an active CMV infection in kidney transplant recipients. Similar results were seen when lymphocytes of kidney recipients were stimulated with PHA before, during and after CMV infection as tested by the AA (section 3.3.2). Stimulation indices were higher before CMV infection and declined with the detection of CMV infection (Fig. 10). Thus it appears likely that CMV infection leads to immunosuppression, measured here as a decline in the T cell response to mitogenic stimulation.

$CD4^+$ T cells regulate both cellular and humoral immune responses *in vivo*, primarily by releasing numerous cytokines (Heinzel *et al.*, 1991; Sad and Mosmann, 1994). $CD4^+$ T cells exist as at least two distinct subsets, Th1 and Th2. Th1 cells are characterized by the production of high levels of IFN-γ, TNF-β and IL-2, whereas Th2 cells preferentially release IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann and Coffman, 1989; Romagnani, 1992). Th1-type cytokines are generally associated with effective defense mechanisms against intracellular

infectious agents by activating macrophages, promoting granuloma formation and inducing delayed type hypersensitivity (DTH) reactions (Mosmann and Coffman, 1989; Romagnani, 1992). Th2-associated cytokines such as IL-4 and IL-10 sometimes allow the progression of intracellular pathogens by down-regulating Th1 responses (Heinzel *et al.*, 1991; Sad and Mosmann, 1994; Romagnani, 1994). It should be pointed out that while both Th1 and Th2 cells produce TNF-α, it is secreted at considerably higher levels in Th1-type responses and that it mediates cytotoxic activity as a part of cell-mediated immunity (Romagnani, 1992). TNF-α also elicits the production of IFN-γ, a Th1-type cytokine. Thus, while TNF-α is not a characteristic Th1-type cytokine, it is of great significance in Th1-type cell-mediated responses.

Immune responses show a broad spectrum of variations according to different infective diseases or autoimmune disorders. These are usually reflected in the patterns of Th1- and Th2-type pattern of cytokine production (Romagnani, 1994). An increase in the level of Th2-type cytokines is usually associated with an enhanced antibody production, allergic disorders and immunopathological reactions to helminthic infestations. On the other hand an elevated level of Th1-type cytokines is closely linked to acute allograft rejection, organ-specific autoimmunity, contact dermatitis and intracellular parasitic infection. Thus, Th1-type cytokines are primarily involved in cell-mediated immunity, while Th2-type cytokines are related to antibody formation. Studies on intracellular pathogens such as HIV (Clerici and Shearer, 1993), murine AIDS (Gazzinelli *et al.*, 1992), vaccinia virus (Actor *et al.*, 1994), herpes simplex virus (Jayaraman *et al.*, 1993) and influenza virus (Graham *et al*, 1994) have shown that Th2 responses generally exacerbate the infection, whereas Th1 responses are clearly protective. Given the strong influence exerted by Th1- and Th2-type responses on the outcome of infections, It was of importance to elucidate Th1- and Th2-type profiles during CMV infection in renal transplantees.

Cytokines play important roles in the outcome of a CMV infection and determining the level and ratio of major cytokines involved in Th1/Th2 paradigm in cell-mediated immunity, may give an insight into components of the association

between CMV infection and cytokine production. The results of this analysis (section 3.4) showed that Th1-type cytokine levels elicited in response to stimulation with a mitogen are lower in kidney recipients with active CMV infections than in those without active CMV infections. Since Th1-type cells provide help for mounting cell-mediated reactions against viral infection, a decline in Th1-type activity may well lead to poor defenses against viruses. At the same time it appears that Th2-type cytokine secretion is not influenced by the CMV status of patients. There was no significant difference in the production of IL-4 and IL-10 between AA-positive and AA-negative kidney transplant recipients (section 3.5). This is consistent with observations in relation to bone marrow transplantation; recipients with active CMV infections had a low level of Th1-type cytokine production while Th2-type cytokine production remained unaltered (Sparrelid *et al.*, 1997). In the latter study the analysis focused on the local production of Th1 and Th2-type cytokines within the lung. This may suggest a common feature in all immunosuppressed transplant patients.

The observation that an ongoing CMV infection (diagnosed by the presence of antigenemia) was closely associated with the production of Th1-type cytokines raised questions about the effect of CMV containing antigen cells on PBMC cultured *in vitro*. Measurement of the proliferative responses and subsequent cytokine production of PBMC stimulated with CMV-infected and non-infected MRC-5 fibroblast cells showed that of 35 kidney recipients with an active CMV infection, diagnosed by the AA, 31(88%) showed poor proliferative responses (S.I.<3) when stimulated with CMV-infected fibroblasts (section 3.7). On the other hand, of 44 kidney recipients without an active CMV infection 40 (91%) responded with positive proliferation (S.I.>3). These results (the low S.I.) indicated that the presence of CMV antigen had a suppressive effect on the proliferation of PBMC. Reduced proliferation of PBMC may be explained in two ways; a) CMV has a direct suppressive effect on lymphocyte responses or b) there is a poor cellular response to CMV.

While the responder PBMC were stimulated with CMV-infected fibroblasts, it is unlikely that these cells served as antigen-presenting cells as fibroblasts are HLA class II-negative. Professional antigen-presenting cells in the responder PBMC may have presented CMV antigens to the T cells.

On the other hand, results obtained with CMV-stimulated PBMC (section 3.7) agreed well with those obtained with PHA-stimulated PBMC when Th1-type cytokines (IFN-γ, TNF-α and IL-2) were produced at significantly higher levels in kidney recipients without an active than with an active infection CMV infection (Essa *et al.*, 2000). Using CMV-infected fibroblasts to test the responses of PBMC, the response was very similar to that of PHA stimulation; PBMC of kidney transplant recipients with active CMV infection produced a significantly lower level of IFN-γ, TNF-α and IL-2 than PBMC obtained from recipients without an ongoing CMV infection (section 3.8). Such a difference was not observed in the case of Th2-type cytokines (IL-4 and IL-10) (section 3.9).

While it is possible that the correlation between CMV-infection and down-regulation of Th1 cytokines is actually due to immunosuppressive therapy, this is unlikely, since both the CMV-infected and uninfected individuals received the same immunosuppressive regimen. It is more likely that CMV infection itself has a selective suppressive effect on Th1-type reactivity in some individuals (Boland *et al.*, 1990). Since cell-mediated immunity in general, and Th1-type immunity in particular is important for protection against CMV infection, the balance between Th1 and Th2-related cytokines may determine the outcome of a CMV infection. Also, it seems that CMV has evolved diverse mechanisms for evading host cellular immune reactions through the inhibition of $CD4^+$ T cell immunosurveillance by disrupting inducible and constitutive production of Th1-type cytokines. This is an advantage for the virus since escaping T lymphocyte immunosurveillance is of critical importance in immunoevasive strategies for intracellular pathogens.

Levels of $CD4^+$ and $CD8^+$ T cell subsets can predict progression to acquired immune deficiency syndrome (AIDS) among human immunodeficiency virus-positive subjects. To investigate whether a similar situation exists in kidney transplant patients, the proportion of lymphocyte phenotype subsets and

granulocytes in the peripheral blood of kidney transplant recipients with and without active CMV infection were investigated. CMV infection is known to induce changes in the lymphocyte subset counts in peripheral blood (Beik *et al.*, 1998, Belles-Isles *et al.*, 1998). Beik et al., (1998) examined changes in the circulating T-cell subsets of renal transplant patients to see if consistent changes occur following CMV infections. Serial blood samples were taken from 28 patients who were on standard triple immunosuppression therapy. Using flow cytometric analysis, the percentage of activated and naive $CD4^+$ and $CD8^+$ T cells were determined. During active CMV infections, percentage of activated $CD4^+$ and $CD8^+$ T cells were increased, while there was a decrease in the percentage of naive $CD4^+$ and $CD8^+$ T cells. Belles-Isles *et al.*, (1998) also quantified the percentage of activated $CD8^+CD38^+$ T cell subsets in 77 kidney transplant patients with CMV disease by flow cytometry. They found that the number of activated $CD8^+CD38^+$ subsets was increased during active CMV infections. In my study, flow cytometric analysis of PMNL from the 35 AA-positive kidney transplant recipients (section 3.10) showed a significant change in the percentage of $CD3^+$ T cells ($p<0.05$) and $CD4^+$ T cells ($p<0.005$) compared with the 44 AA-negative kidney transplant recipients. This may be related to immunologic abnormalities that predispose renal transplant recipients to CMV infection. On the other hand, the percentage of $CD8^+$ T cells remains the same between the CMV-positive and CMV-negative kidney transplant recipients (Fig. 21). This data probably reflects that there are proportionally more $CD8^+$ T cell within the $CD3^+$ population in the CMV-infected than the uninfected population. It is difficult to compare our results with that of Beik *et al.*, 1998 and Belles-Isles *et al.*, 1998 which is due to the fact that we did not determine the percentage of activated and naïve $CD4^+$ and $CD8^+$ T cells separately rather we quantified the percentage of total $CD4^+$ and total $CD8^+$ T cells. On the other hand, the significant reduction in the percentage of $CD4^+$ T cells in the AA-positive group may play an important role in the reduced proliferative responses of the PBMC to PHA and CMV-antigen.

As mentioned earlier, (section 1.5.2.2) CMV has evolved mechanisms to evade cytotoxic T cell recognition of infected cells by down-regulating Class I

molecules (Barnes and Grundy, 1992). Thus, as infection progresses, the infected cell loses its ability to present viral peptides to cytotoxic T cells via the class I pathway, and becomes refractory to lysis. This may explain the relatively small, and statistically insignificant change in the proportion of $CD8^+$ T cells present in kidney transplant recipients with an ongoing CMV infection when compared to the recipients without an active CMV infection. Polymorphonuclear leukocytes (PMNL) and mononuclear cells are the principal subsets that express viral antigens during CMV infection. In general, more than 95% of all antigen-positive leukocytes belong to the PMNL fraction. Patient samples were analyzed for other cellular subsets of potential relevance in the development of CMV infection. No significant difference in the percentage of granulocytes, T cells, B cells and activated T cells subsets between AA-negative and AA-positive kidney transplant recipients could be seen. Similarly, analysis did not reveal any alteration in the percentage of $CD15^+$, $CD2^+$, $CD26^+$, $CD19^+$ lymphocyte subsets.

The proportion of NK cells did not differ significantly between the AA-positive and AA-negative kidney transplant recipients (section 3.7) possibly suggesting that NK cells did not recognize CMV-infected cells. However, CMV infected cells *in vitro* have been shown to be killed by NK cells (Borysiewicz *et al.*, 1986; Starr and Garrabrant, 1984). As mentioned earlier, (section 1.5.2.3) *in vivo*, CMV may have evolved a mechanism for evading the killing activity of infected cells by NK cells by encoding a class I HLA homologue, the UL-18 gene product (Beck and Barrell, 1988; Chee *et al.*, 1990), which, when transfected into cells, was able to bind to the CD94 complex on NK cells. This provided a strong inhibitory signal preventing the lysing activity of natural killer cells (Reyburn *et al.*, 1997). This may explain why no significant difference in the percentage of NK cells of the kidney transplant recipients according to their CMV antigenemia status could be seen.

The immunological mechanisms that predispose to CMV infection are poorly understood. The analysis of kidney transplant recipients indicates that an active CMV infection is characterized by decreased lymphocyte proliferative responses, decreased levels of Th1-type cytokines (IFN-γ, TNF-α and IL-2), low

number of $CD4^+$ and $CD3^+$ lymphocytes. These alterations may contribute to or be part of the inadequate immunological responses that predispose the development of CMV disease in renal transplant recipients. Although larger studies are needed to investigate whether this observation may be exploited in a clinically useful manner, the data indicate that further studies on the immune defense mechanisms against CMV infections in humans are required.

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Th1-Type Cytokines Production Is Decreased in Kidney Transplant Recipients With Active Cytomegalovirus Infection

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Cytomegalovirus (CMV) infection is a major complication after kidney transplantation. Despite antiviral therapy the infection contributes significantly to high morbidity. The present study was aimed at determining: (a) the stimulation index (S.I.) of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) and (b) the levels of Th1- and Th2- related cytokines in kidney transplant recipients with and without active CMV infection. Thirty-five patients with, and 44 without active CMV infections, as diagnosed by a CMV antigenemia assay, were inducted into this study. After PHA stimulation of PBMC from patients, stimulation index (S.I.) was determined by radioactive thymidine uptake while the production of Th1-type cytokines (interleukin-2 [IL-2], interferon-γ [IFN-γ], and tumor necrosis factor-α [TNF-α]) and Th2-type cytokines (IL-4, IL-10) were measured by enzyme-linked immunosorbent assay. PBMC of patients with active CMV infection showed significantly lower S.I. values than patients without an ongoing CMV infection ($P < .0001$). Levels of Th2-type cytokines in CMV-infected and uninfected kidney recipients were similar; however, the levels of the Th1-type cytokines were significantly lower in CMV-infected patients. Low levels of Th1-type cytokines seem to correlate well with active CMV infection in kidney recipients. ***J. Med. Virol. 60:223–229, 2000.***

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KEY WORDS: viral infection; immunosuppression; T helper cells

INTRODUCTION

Although cytomegalovirus (CMV) infection in immunocompetent individuals usually causes only mild or subclinical disease, in immunocompromised patients it may cause severe clinical symptoms [Meyers et al., 1986]. However, the mechanisms of tissue injury caused by CMV infection in renal transplant recipients are not well understood. Studies in immunosuppressed humans and animals have revealed that $CD4^+$ and $CD8^+$ T cells are of crucial importance in the maintenance of immunity to CMV as well as for the eradication of an ongoing infection [Borysiewicz et al., 1988; Jonjic et al., 1994]. In human and animal experiments, the adoptive transfer of syngeneic, polyclonal $CD8^+$ T cells to immunosuppressed individuals has been shown to provide protection from CMV disease [Quinnan et al., 1982; Reusser et al., 1991]. Adoptive immunotherapy with CMV-specific $CD8^+$ T cell clones from allogeneic donors has been shown to prevent the development of CMV disease [Reddehase et al., 1985, 1987]. In addition, allogeneic transfer of CMV-specific $CD8^+$ cell clones has been found to reconstitute cellular immunity against CMV in vivo [Riddell et al., 1992]. It has also been shown that infusion of increasing doses of cells results in an increased CMV-specific cytotoxic T lymphocyte response in recipients [Walter et al., 1995].

It is clear that the Th1 and Th2 subsets are of major importance in determining the class of immunoprotective function in infectious diseases. Thus a bias either toward Th1-dominance or Th2-dominance can make a difference between effective elimination of the virus and exacerbation of the disease [Mosmann and Sad, 1996]. Research on HIV infection [Clerici and Shearer, 1993], murine AIDS [Gazzineli et al., 1992], vaccinia virus [Actor et al., 1993], herpes simplex virus [Jayaraman et al., 1993] and influenza virus [Graham et al., 1994] has shown that generally Th2 responses exacerbate the infection, whereas Th1 responses are protective. Given the strong influence exerted by Th1- and Th2-type immunity on the outcome of infections, we

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considered it of importance to elucidate Th1- and Th2-type profiles in CMV infection in renal transplantees.

Because the proliferative response of T lymphocytes to mitogens generally reflects the status of cellular immunity, we have measured the stimulation index (S.I.) values of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from kidney recipients with and without active CMV infection. Culture supernatants of PHA-stimulated PBMC were analyzed for Th1-type and Th2-type cytokines, to determine the status of Th1-Th2 profiles.

MATERIALS AND METHODS

Study Population

Seventy-nine kidney transplant patients (31 women, 48 men) who received kidney transplants during 1996 and 1997 were included in this study. Their ages ranged from 18 to 60 years; most subjects were 30–45 years old (median age = 35 years). All patients received an immunosuppressive regimen of cyclosporin A, azathioprine, and steroids. Of the 35 patients positive for the CMV-pp65 antigen, 32 had one or more of the following symptoms associated with CMV infection/disease: fever, arthralgia, leukopenia, thrombocytopenia, pneumonitis, hepatitis, retinitis, and gastrointestinal ulceration. Diagnosis of CMV infection/disease conformed to the guidelines suggested in the Workshop on CMV Disease [Ljungman and Plotkin, 1995]. Patients in this study had to have at least one of the CMV-associated symptoms in addition to being antigenemia-positive to be considered as having CMV disease. Serial samples were collected at different intervals after transplantation and patients were monitored for 6 months. Blood samples were collected in two ethylenediamine tetraacetic acid (EDTA)-containing tubes and transferred to the laboratory within 2–3 hr. Antigen detection and mitogen-induced activation of PBMC were carried out on the same day.

CMV Antigenemia Assay (AA)

A total of 5 ml blood was collected from each patient and processed immediately. Leukocytes were isolated by the dextran sedimentation method. Following incubation and centrifugation, the cell pellet was suspended in phosphate-buffered saline (PBS) and the erythrocytes were lysed with 0.8 mM ammonium chloride. The remaining cells were centrifuged, washed in PBS, counted and spotted onto glass slides (50,000 cells per spot). They were then dried and fixed in acetone-methanol, stained with CMV-vue Kit (Incstar, Inc., Stillwater, MN) according to the manufacturer's recommended procedure for immunoperoxidase staining. Numbers of cells containing the CMV-specific pp65 antigen were counted under a light microscope. Patients with ≥ 5 cells containing the CMV-specific pp65 antigen out of 50,000 cells were considered to be positive for AA. Each patient was tested three or more times by the AA at different time points. Patients who tested positive at the time of mitogen-induced stimulation were considered AA-positive in this study.

Mitogen-Induced Stimulation of PBMC

PBMC from 35 antigenemia-positive and 44 antigenemia-negative transplant recipients were obtained by Ficoll-Hypaque (Pharmacia Biotech, Sweden) density gradient centrifugation of peripheral blood at $1500 \times g$ for 20 min. PBMC were suspended in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum, aliquoted into 96-well tissue culture plates at a density of 10^5 cells per well, and then stimulated with PHA at a concentration of 5 μg/ml for a period of 96 hr. The concentration of 5 μg/ml was chosen on the basis of optimal proliferation in our laboratory. The supernatants from some of the wells were harvested 24 and 96 hr later, while some of the wells were pulsed with [^{3}H] thymidine (1 μCi per well) at 72 hr for assessment of mitogen-induced proliferation. Thymidine-pulsed wells were harvested 18 hr later and the radioactivity estimated. The S.I. was calculated as a ratio of thymidine uptake by PHA-stimulated cells to that by nonstimulated cells. Background thymidine uptake in the absence of PHA was in the range of 80–500 cpm. Samples yielding S.I. of less than 50 were considered as proliferation-negative. The cut-off of 50 was based on experience in our Clinical Immunology Laboratory that an S.I. less than 50 generally indicates low proliferation due to poor immune status of the subjects.

Assay for Cytokines

Levels of the Th1-type cytokines, interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), and the Th2-type cytokines, IL-4 and IL-10, were estimated in the supernatants of PBMC of 44 CMV antigenemia-negative kidney recipients and 35 antigenemia-positive recipients stimulated with PHA for 24 (for IL-2) and 96 hr (for TNF-α, IFN-γ, IL-4, and IL-10). Th1-type cytokines and Th2-type cytokines were assayed by enzyme-linked immunosorbent assay (ELISA) using kits obtained from Immunotech SA (France). These consisted of sandwich type ELISA; briefly, the first step led to the capture of the relevant cytokines by monoclonal anti-cytokine antibodies bound to the wells of microtiter plates. In the second step, biotinylated monoclonal antibody was added together with streptavidin-enzyme (peroxidase or alkaline phosphatase) conjugate. The biotinylated antibody binds to the antibody-antigen complex, and in turn, binds the conjugate. After incubation, the wells were washed and the binding of streptavidin-enzyme via biotin was followed by the addition of a chromogenic substrate. The intensity of the coloration produced is proportional to the concentration of the cytokine present in the sample. The sensitivity of each of the assays was as follows: 5 pg/ml for IL-2, 10 pg/ml for TNF-α, 3 pg/ml for IFN-γ, 5 pg/ml for IL-4, and 5 pg/ml for IL-10.

Statistical Analysis

The standard Mann–Whitney test was used for nonparametric comparisons of median cytokine levels.

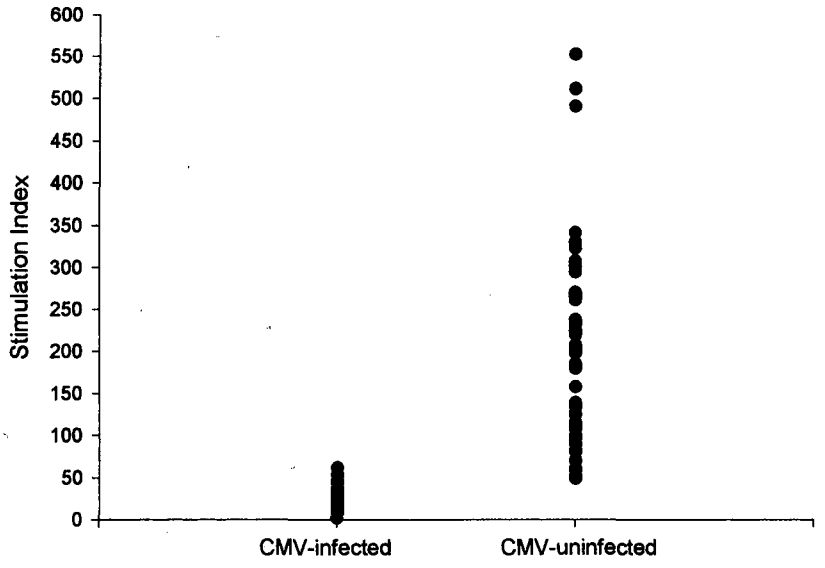

Fig. 1. Stimulation indices (S.I.) of 35 cytomegalovirus (CMV)-infected and 44 CMV-uninfected kidney recipients.

RESULTS

PHA-Induced PBMC Proliferation

Figure 1 shows the stimulation indices of PBMC from antigenemia-positive and antigenemia-negative kidney transplant recipients after stimulation with PHA. It was found that the indices in the antigenemia-positive kidney recipients was inversely related to the degree of antigen positivity ($r^2 = 0.052$, Fig. 2); in other words, patients who were highly antigenemia positive had lower stimulation indices than were patients who had low antigenemia. In the group of antigenemia-positive recipients, the number of CMV-pp65-positive cells varied between 10 and 500 and the S.I. was as low as 2 when the number of CMV-pp65-positive cells was 500; the S.I. was as high as 50 when the number of CMV-pp65-positive cells was around 10. As can be seen from Table I, of the 35 antigenemia-positive patients 33 (94%) were proliferation negative. On the other hand, of the 44 antigenemia-negative patients only 1 was unresponsive to PHA (2%). Figure 3 shows the stimulation indices of PBMC from antigenemia-positive kidney recipients before the detection of active CMV infection, during active CMV infection, and after the recovery from active CMV infection. It was found that the indices in the antigenemia-positive kidney recipients were decreased during active CMV infection (mean S.I. = 24, $P < .0005$) when compared with the indices in the same kidney recipients before active CMV infection (mean S.I. = 80, $P < .0005$). It was also found that the indices in these recipients are marginally increased, but still low after loss of antigenemia (mean S.I. = 28, $P < .05$).

Th1 Cytokines

The levels of IL-2, TNF-α, and IFN-γ were consistently higher in kidney recipients without an active CMV infection than in the group of recipients with an active CMV infection (Fig. 4). These differences were statistically significant in the case of IL-2 ($P < .05$) at 24 hr, and IFN-γ ($P < .0005$) and TNF-α ($P < .005$) at 96 hr. P values were calculated by the Mann–Whitney test conducted on median cytokine levels. The median pg/ml values (range) for IFN-γ, TNF-α, and IL-2 in the antigenemia-positive patients are 7 (0–321), 311 (0–6893.9), and 447 (0–5136), respectively; on the other hand, in the antigenemia-negative patients, the median pg/ml values are 131 (0–815.6), 2213 (0–7074), and 1501 (0–7696), respectively. It is evident that kidney recipients with an active CMV infection produce lower levels of Th1 cytokines than do the kidney recipients without an active CMV infection. Therefore, these data suggest that CMV infection impairs the production of Th1-related cytokines.

Th2 Cytokines

The levels of IL-4 and IL-10 were measured in antigen-positive and antigen-negative patients. There were no statistically significant differences in the levels of IL-4 and IL-10 between antigenemia-positive and -negative groups (Fig. 5).

Th1/Th2 Cytokines Ratios

The ratio of Th1 to Th2 cytokines in a given sample is considered to be of greater significance than the levels of cytokines alone. Therefore, we calculated the ratios of different Th1 to Th2 cytokines produced by PHA-stimulated PBMC. The mean values of Th1 cytokines were compared with that of Th2 cytokines produced by PBMC of kidney recipients with and without active CMV infections. The difference between the two groups in some of the Th1:Th2 ratios was found to be striking. Table II depicts the ratios of Th1 to Th2 cytokines for the two time points tested. For example, the IL-2:IL-4 ratio at 24 hr and TNF-α:IL-4 ratio at 96 hr were lower in the antigenemia-positive group as compared with the antigenemia-negative group, indicating a lower Th1-bias in the antigenemia-positive group. In some other cases the differences in ratios were not as striking, even though the ratios were consistently lower in

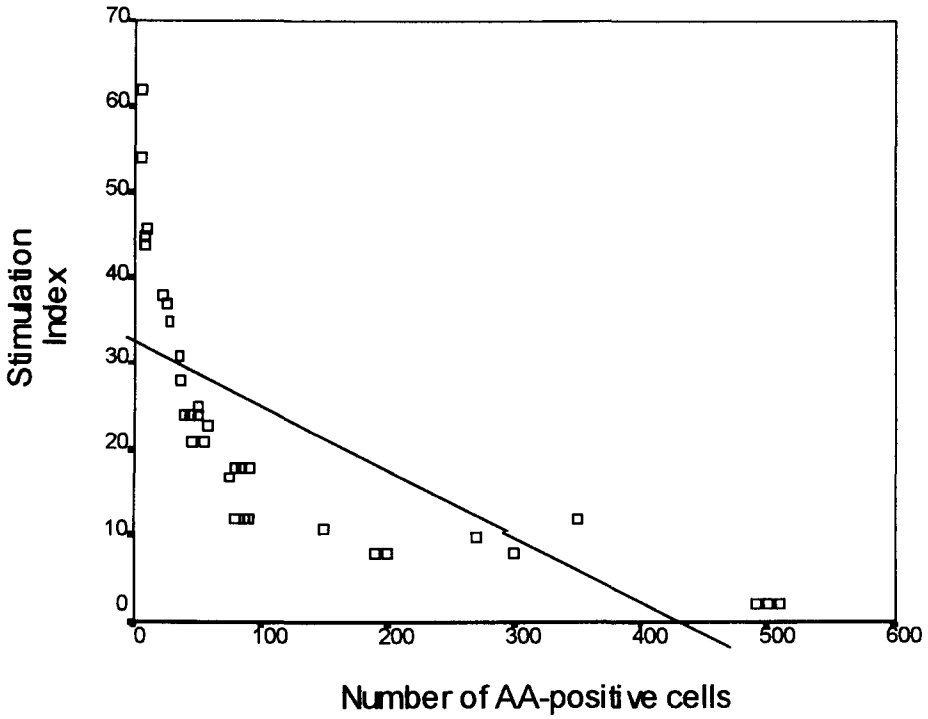

Fig. 2. Correlation between lymphocyte proliferation (stimulation index) in response to mitogenic stimulation and the number of antigenemia assay (AA)-positive cells in kidney transplant recipients.

TABLE I. Mitogen-Induced Proliferation* of PBMC in CMV-Infected and -Uninfected Transplant Recipients

	Proliferation positive	Proliferation negative	Total
CMV infected	2	33	35
CMV uninfected	43	1	44
Total	45	34	79

PBMC, peripheral blood mononuclear cells; CMV, cytomegalovirus; S.I., stimulation index.
*PBMC proliferation was considered positive when the S.I. $\geq$ 50.

infected individuals. Thus, general trend supports a lower production of Th1 cytokines by the antigenemia-positive group than by the antigenemia-negative group.

DISCUSSION

For the prevention of an active CMV infection and CMV disease, cellular immunity in general, and the action of cytotoxic T cells to CMV-infected target cells in particular, is the most important factor in the host defense mechanisms [Rook et al., 1984; De Waal Malefyte et al., 1993]. In kidney recipients the outcome of CMV infection depends to a great extent on the activity of T cells [Mosmann and Coffman, 1989], and therefore PBMC proliferation to PHA was used as an indicator of the T-cell responses in kidney transplant recipients with and without CMV infections.

Results of PHA-induced proliferative responses of PBMC revealed that recipients with an active CMV infection had lower levels of proliferation (S.I. < 50) as compared with recipients without active CMV infections (S.I. $\geq$ 50). These results show that the reduction in the PHA-induced response of PBMC correlates well with the development of an active CMV infection. This finding illustrates the importance of helper-T-cell responses, and fits in with the well-recognized role of CMV-specific cytotoxic T cells in recovery from opportunistic CMV infections [Quinnan et al., 1982; Reusser et al., 1991]. On the other hand, the diminished T-cell response may be due to the immunosuppressive effect of active CMV infection in kidney transplant recipients [Rook et al., 1984]. The data (Fig. 3) indicate that stimulation indices are higher before CMV infection and declines with the detection of CMV infection. Thus it appears likely that CMV infection leads to immunosuppression, reflected here as a decline in the T-cell response to mitogenic stimulation. Interestingly, this immunosuppression is also reflected by a decline in the production of Th1-type cytokines.

$CD4^+$ T cells regulate both cellular and humoral immune responses in vivo, primarily by releasing numerous cytokines [Heinzel et al., 1991; Sad and Mosmann, 1994]. $CD4^+$ T cells exist as at least two distinct subsets, the Th1 and Th2. Th1 cells are characterized by the production of high levels of IFN-γ, TNF-β, and IL-2, whereas Th2 cells preferentially release IL-4, IL-5, IL-10, and IL-13 [Mosmann and Coffman, 1989; Romagnani, 1992]. Th1-type cytokines are generally associated with effective defense mechanisms against intracellular infectious agents by activating macrophages; promoting granuloma formation and inducing delayed type hypersensitivity (DTH) reactions. Th2-associated cytokines such as IL-4 and IL-10 sometimes allow the progression of intracellular infectious diseases by down-regulating Th1 responses [Heinzel et al., 1991; Sad and Mosmann, 1994]. It should be pointed out that

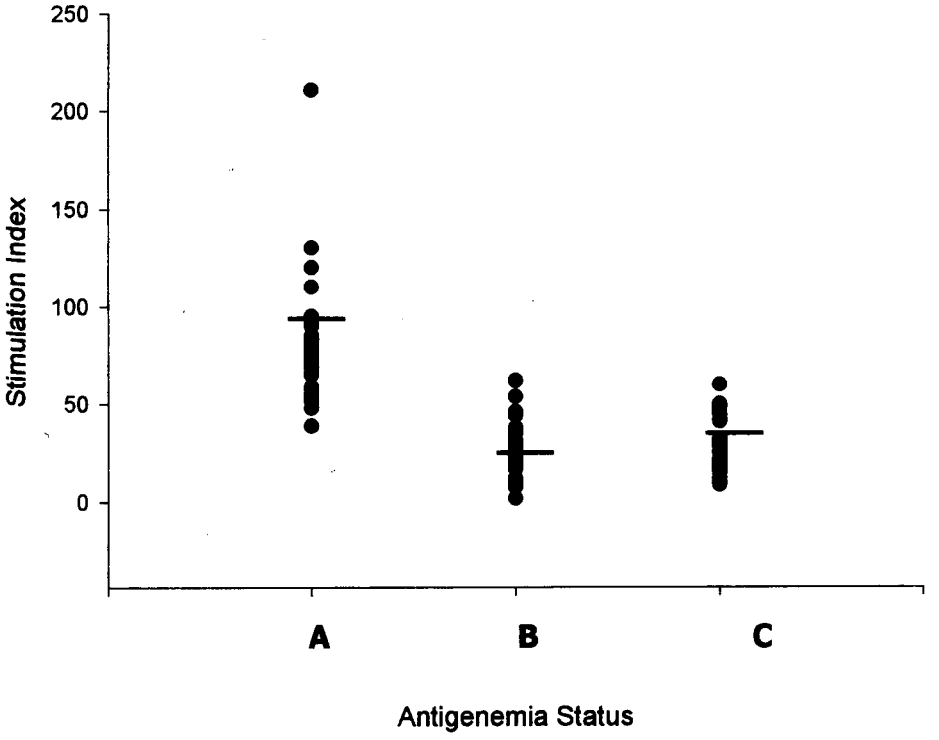

Fig. 3. Stimulation indices in (A) kidney transplantees before cytomegalovirus (CMV) infection as tested by the antigenemia assay, (B) during infection, and (C) after infection as indicated by a negative antigenemia assay.

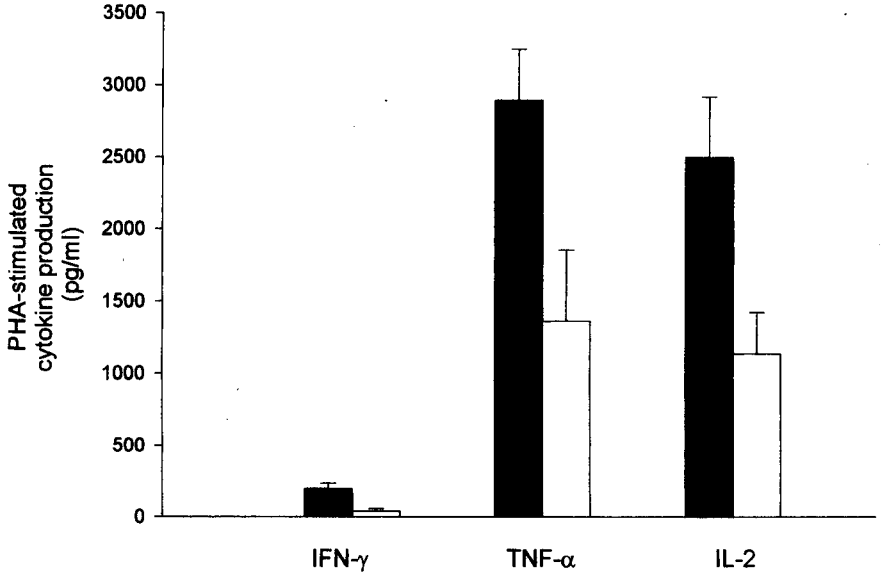

Fig. 4. Levels of Th1-type cytokines produced by mitogen-induced peripheral blood mononuclear cells (PBMC) after 24 hr of culture for interleukin-2 (IL-2) and 96 hr of culture for interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Dark bars represent mean levels (±SEM) of the cytokines in cytomegalovirus (CMV)-negative individuals; white bars represent mean levels in CMV-positive subjects.

whereas both Th1 and Th2 cells produce TNF-α, it is secreted at higher levels in Th1 responses [Romagnani, 1992] and that it mediates cytotoxic activity as a part of cell-mediated immunity. TNF-α also elicits the production of IFN-γ, a Th1-type cytokine. Thus, although TNF-α is not a characteristic Th1-type cytokine, it is of great significance in Th1-type cell-mediated responses.

This study shows that Th1 cytokine levels elicited in response to stimulation with a mitogen are lower in kidney recipients with active CMV infections than in those without active CMV infections. Because Th1 cells provide help for cell-mediated reactions against viral infection, a decline in Th1 activity may well lead to poor defenses against viruses. Furthermore, a decline in Th1-type activity may adversely affect the outcome of adoptive immunotherapeutic transfer of virus-specific T cells. Studies on the adoptive transfer of CMV-specific clones, for example, are currently underway [Riddell and Greenberg, 1997]; the present data on depressed Th1-type activity may be relevant in this regard. There appears to be no significant difference in Th2 cytokine secretion between CMV-positive and CMV-negative patients. Similar observations have been published [Sparrelid et al., 1997] in relation to

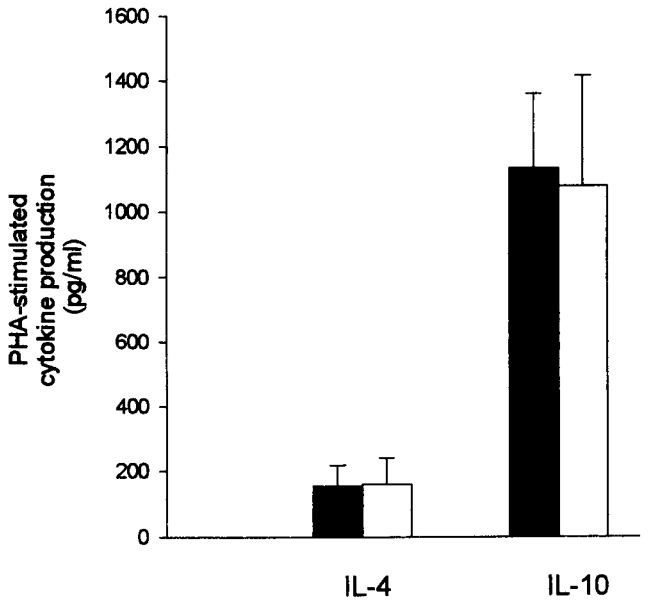

Fig. 5. Levels of Th2-type cytokines secreted by mitogen-induced peripheral blood mononuclear cells (PBMC) after 96 hr of culture. Dark bars represent mean levels (±SEM) of the cytokines in cytomegalovirus (CMV)-negative individuals; white bars represent mean levels in CMV-positive subjects.

TABLE II. Th1:Th2 Cytokine Ratios in CMV-Infected and -Uninfected Transplant Recipients*

Th1:Th2 ratio	CMV uninfected	CMV infected
After 24 hr of culture		
IL-2:IL-4	16.0	7.0
IL-2:IL-10	2.2	1.1
After 96 hr of culture		
TNF:IL-4	18.6	8.4
TNF:IL-10	2.6	1.3
IFN:IL-4	1.3	0.2
IFN:IL-10	0.1	0.04

*CMV, cytomegalovirus; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

bone marrow transplantation; recipients with active CMV infections had a low level of Th1 cytokine production whereas Th2 cytokine production remained unaltered. Though that study dealt with local production of Th1 and Th2 cytokines within the lung, the results are similar to our findings.

It is possible that the correlation between CMV infection and down-regulation of Th1 cytokines is actually due to immunosuppressive therapy. However, this explanation is unlikely, because both the CMV-infected and uninfected individuals received the same immunosuppressive regimen. It is more likely that CMV infection itself has a selective suppressive effect on Th1-type reactivity [Boland et al., 1990]. Because cell-mediated immunity in general, and Th1-type immunity in particular, is important for protection against CMV infection, the balance between Th1-related and Th2-related cytokines may determine the outcome of a CMV infection.

The data presented above indicate the importance of Th1-type cytokines in the pathogenesis of CMV infection.

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The use of flow cytometry for the detection of CMV-specific antigen (pp65) in leukocytes of kidney recipients

Essa S, Pacsa AS, Al-Attiyah R, El-Shazly A, Raghupathy R, Said T. The use of flow cytometry for the detection of CMV-specific antigen (pp65) in leukocytes of kidney recipients.
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Abstract: Flow cytometric assay (FCA) was used to detect a cytomegalovirus (CMV) specific antigen (pp65) in CMV-infected fibroblast cells and in leukocytes of kidney recipients. FCA distinguished clearly between the infected and non-infected fibroblast cells. Regarding transplant patients, the FCA was positive when the number of antigenemia assay (AA) positive cells was five or more per 5×10^4. Moreover, the percentage of antigenemia-positive cells by FCA correlated well with symptomatic CMV infections.

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Key words: CMV – flow cytometry – pp65 assay – renal transplants

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Cytomegalovirus (CMV) infection has been recognized as a significant cause of morbidity and mortality in bone marrow, cardiac, and renal transplant patients as well as in patients infected with human immunodeficiency virus (HIV) (1–5). Traditionally, CMV infection is detected either by virus isolation or by the demonstration of characteristic inclusion bodies in biopsy specimens (6). Serological testing is also used to confirm exposure to CMV, but it is not reliable since immunosuppressed patients may not have a change in antibody titer or produce CMV-IgM in case of an active CMV infection (2, 7). Among the different techniques used to confirm an active CMV infection is the detection of the CMV-specific early antigen (pp65). This antigenemia assay (AA) has the advantage of quantifying the viral load according to the number of antigen-containing cells, which correlates well with manifestations of CMV disease (8, 9). However, the quantification of antigen-positive cells by the AA is subjective and demands very careful and time-consuming microscopic examination (8). With the advent of effective antiviral chemotherapy for human CMV infection, more rapid and sensitive techniques are required to identify CMV infection during the acute stage of the illness and also to monitor antiviral therapy (10, 11). The use of flow cytometry in the detection of viral infections has grown to significant proportions (12–16). Flow cytometry is an advanced technology, which can be used to analyze a large number of specifically labeled cells in a quantitative manner. In this study, we looked at the possibility of using flow cytometry for rapid and accurate detection of CMV-specific antigens in clinical samples for the early detection of CMV-infected cells in transplant patients.

Here we present the results of the flow cytometric assay (FCA) for direct quantification of CMV-infected cells in human embryonic fibroblast cultures (MRC-5) and in polymorphonuclear leukocytes (PMNLs) of transplant patients.

Materials and methods

Cell line

MRC-5 fibroblast cell cultures were propagated in minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in 50 cm^2 size flasks at 37°C in the presence of 5% CO_2.

Virus

The AD169 strain of human CMV obtained from the American Type Culture Collection was propagated in our laboratory. The MRC-5 cells were infected with a multiplicity rate of 0.1 virus/cell and, after a 30 min absorption period, fresh medium was added and the incubation started. This was considered 0 time.

Trypsinization and fixation of CMV-infected cell culture

Cells were removed with a 0.25% trypsin solution. After detachment (5–10 min at 37°C), cells were suspended in 2 mL of 10% MEM, washed by centrifugation (2 × in PBS), then resuspended in 1 mL PBS and counted. The number of cells was adjusted to 1.0–1.5 × 10^6 cells/mL and stored in 90% methanol at −20°C and stained within < 24 h for FCA.

Study population

Seventy-nine kidney transplant patients (31 female, 48 male) who received kidney transplants during 1996 and 1998 were inducted into this study. Their ages ranged from 18 to 60 yr, the majority being 30–45 yr old with a median age of 35. All patients (35 AA-positive and 44 AA-negative) received an immunosuppressive regimen of cyclosporin A, azathioprine, and steroids. Of the 35 patients positive for the CMV-pp65 antigen, 32 had one or more of the following symptoms associated with CMV infection/disease: fever, arthralgia, leukopenia, thrombocytopenia, pneumonitis, hepatitis, retinitis, and gastrointestinal ulceration. Diagnosis of CMV infection/disease conformed to the guidelines suggested in the Workshop on CMV disease (17). Patients in this study had to have at least one of the CMV-associated symptoms in addition to being antigenemia-positive in order to consider them as having CMV disease. Serial samples were collected at different intervals after transplantation and patients were monitored for 2–12 wk. Blood samples were collected in EDTA containing tubes and transferred to the laboratory within 2–3 h. All samples were processed on the same day. For both AA and FCA only one sample for each patient was included in this study. If the patient was AA-positive during the period of monitoring only the samples with the highest number of AA-positive cells were tested also for FCA.

Isolation of PMNLs

Blood samples taken from the patients were processed immediately to isolate PMNLs by using the dextran sedimentation method as previously described (16). Briefly, following the incubation of blood with 5% dextran at 37°C for 10–15 min, the leukocyte-rich layer was transferred to a test-tube and centrifuged for 10–11 min at 300 *g*. The pellet was then suspended in a RBC Lysing Medium and incubated for 4–6 min at room temperature. PMNLs were then washed twice with PBS, counted and adjusted to 2 × 10^6 cells/mL with cold PBS and the cells were divided into two parts. One part of the cells was fixed with 1% paraformaldehyde for 15 min at 4°C, washed once with PBS, resuspended in cold 80% methanol (2 parts PBS + 8 parts MEOH) and kept at −20°C. FCA was performed within < 24 h, while the other part was used immediately for the AA.

Antegenemia assay

AA was performed as described by the manufacturer (CMV-vue Kit; INCSTAR Corporation, Stillwater, MN). Briefly, 5 × 10^4 cells/25 μL were added to two test wells on the CMV-vue microscope slide. Cells were then fixed with acetone for 10 min and allowed to air dry. Then, 25 μL of a monoclonal antibody to CMV lower matrix, early structural protein (pp65) was added to control and test wells and incubated for 45–50 min at 37°C in a moist chamber. The slides were then incubated with 25 μL of the conjugate (anti-mouse IgG labeled with peroxidase) at 37°C for 45–50 min. At the end of the incubation period, slides were immersed into substrate solution for 10–11 min at room temperature and washed with PBS. After mounting the slides, control and sample wells were examined with brightfield microscopy at 200 × – 400 × magnification. Prominent red–brown nuclear staining indicated the presence of the antigen.

FCA

Fixed and permeabilized cells (MRC-5 or PMNLs) were stained as previously described (14, 16) using mouse monoclonal antibodies (C10/C11; Clonab

CMV, Biotest, Landsteinerstrasse 5, Dreieich, Germany), specific to the CMV lower matrix, early structural (pp65) antigen. Briefly, after removing the fixative by centrifugation (1000 *g* for 5 min), cells were washed in PBS containing 20% AB serum (PBS/AB). Cells were then resuspended in 200 µL of the monoclonal antibody to pp65 diluted 1:5 in PBS/AB. For each test sample, a control sample was included using an isotypically matched monoclonal antibody (Caltag/Tebu, Marnes la Coquette, France). Both tubes were then incubated at 37°C for 60 min. After washing and centrifugation (2 × in PBS), the second antibody which is FITC-conjugated goat-anti-mouse immunoglobulin (Biotest, Breieich, Germany) diluted at 1:20 was added and incubated at 37°C for 60 min. This was followed by two rinses in PBS/AB and the cells were resuspended in 200 µL of PBS/AB and analyzed by an EPICS-Profile II (Coulter Electronics, Luton, United Kingdom) flow cytometer.

Controls used: mock-infected MRC-5 fibroblast cells, PMNLs from patients without active CMV infections served as negative controls. MRC-5 cells infected with the AD169 reference strain (at a multiplicity of infection between 0.01 and 0.1) served as positive control.

Fluorescence intensity of the cells was analyzed with a 488-nm air-cooled argon laser. The instrument was calibrated daily by using various quality control reagents. Data acquisition was triggered by cell size (forward versus 90° scatter). The green fluorescence was filtered through a 530/30 band pass absorption filter. At least 50000 PMNLs or 10000 MRC-5 fibroblasts were gated by light scatter (PMNLs gates were assessed for accuracy when appropriate by using CD15 staining (Immunotech, Marseille, France), debris and dead cells were excluded from the analysis by the conventional scatter gating method. All data were expressed in log fluorescence histogram form using a region defined according to isotype control analysis.

Statistical analysis

For calculating the positive predictive value (PosPV) and the negative predictive value (NegPV) the following formulas were used: PosPV = (number of patients with positive test results and CMV-related disease/total patients with positive test results) × 100. NegPV = (number of patients with negative test result and without CMV-related symptoms/total patients with negative test results) × 100. Calculation of the mean, standard deviation (± SD) values and correlation were carried out using an SPSS software package.

Results

Establishment of a model cell culture system for standardizing flow cytometry for the detection of CMV-specific antigen

It was shown that the percentage of cells expressing the pp65 antigen increases proportionally with time after infection. On day 1 post-infection, 51% of the cells expressed the antigen. That was followed by gradual increase until it reached 90% on day 7. Mean fluorescence intensity (FI) values also correlated with the percentage of infected cells. Furthermore, FCA gave a complete separation between uninfected and infected MRC-5 cells.

Detection of CMV-specific pp65 antigen in PMNLs by antigenemia and flow cytometry assays

For the sake of comparison, 79 samples were tested by both assays (AA and FCA). The following results were obtained by the AA: 12 patients had 5–20, 9 patients had 21–50, 8 patients had 51–200 and 3 more than 200 AA-positive cells/5 × 10^4 (Table 1). FCA resulted in the following: all of the AA-negative patients (n = 47) were also negative, samples with 5–20 AA-positive cells per 5 × 10^4 proved to be positive in FCA with 0.16% mean fluorescence. As Table 1 shows, the mean % of fluorescence increases proportionally with the

Table 1. Number of antigen-positive PMNLs as determined by AA and FCA

Patients tested	AA-positive cells[a]	FCA fluorescence[b]	FCA mean FI value[c]	CMV-related clinical symptoms
47	0–4	0.01 (± 0.01)	0.4	No symptoms, AA-negative
12	5–20	0.6 (± 0.02)	0.8	No symptoms
9	21–50	1.8 (± 0.02)	3.6	Fever, leukopenia
8	51–200	3.5 (± 0.04)	5.8	Fever, leukopenia
3	>200	7.8 (± 0.05)	11.8	Fever, leukopenia, hepatitis and elevated liver enzymes

[a] AA is considered as positive when five or more pp65 positive cells per 50 000 are detected.
[b] Results are expressed as mean of % ± SD.
[c] Mean FI values were obtained by gating on the population of interest and calculating the mean FI.

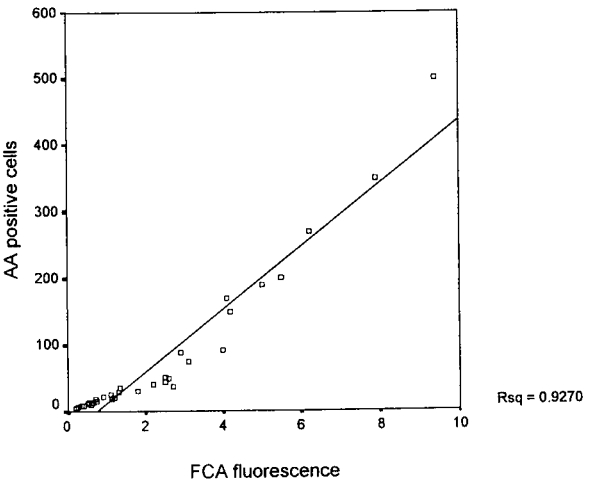

Fig. 1. Correlation between FCA fluorescence and the number of AA-positive cells in 35 kidney transplant recipients.

number of AA-positive cells to reach 3.5% when the number of AA-positive cells was >200. Figure 1 shows a strong correlation between AA and FCA. Regarding CMV-related clinical manifestations FCA had a negative predictive value of 97.5% while its positive predictive value was 82.4%. Since the determination of the number of CMV-infected cells by the AA is a rather cumbersome and subjective methodology, FCA may offer an alternative, being a much faster and objective method for detecting CMV antigen-positive cells by screening a larger number of cells with a considerable accuracy. Therefore, the FCA has the potential to diagnose an on-going CMV disease and to monitor the effect of antiviral therapy in kidney recipient.

Discussion

Since flow cytometer analysis offers a rapid and reliable quantification of cells stained with fluorochrome-labeled antibodies, it has the potential for the diagnosis of CMV infection in transplant patients. However, prior to its use for diagnostic purposes, it should be standardized and compared to an established method. Indeed, parameters of FCA can be tested with human diploid fibroblast cells infected with CMV. In this system FCA performed well by detecting fibroblast cells stained with FITC-labeled monoclonal antibodies specific to CMV-related early matrix protein (pp65). A comparison with the so-called CMV AA showed that the FCA has approximately the same sensitivity as that of the AA. This result was encouraging to use the FCA for the detection of pp65-antigen in leukocytes of kidney recipients. In samples of kidney recipients, FCA detected the pp65 antigen even when the number of AA-positive cells was between 5 and 20. This indicates that FCA is as sensitive as the AA.

These results show that FCA detects the CMV pp65 antigen in PMNLs of kidney recipients with active CMV infection. The definite advantage of the FCA is that a large number of cells can be screened in a short time with accuracy. This may enhance the validity of the results. These data show that the FCA has the potential to be used for the diagnosis of on-going CMV infections. It seems to be a valuable approach for monitoring the effect of antiviral therapy in transplant patients.

Further studies should be carried out to study the effect of fixation and storage on the performance of FCA. This method may also be useful for the detection of other pathogens, where specific monoclonal antibodies are available.

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Diagnosis of Cytomegalovirus Infection by the Detection of Early Antigen (pp65) in Leukocytes of Kidney Transplant Patients

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Key Words
Cytomegalovirus · Antigenemia assay · Diagnosis · Kidney recipients

Abstract

Objective: Cytomegalovirus (CMV) infection is a frequent complication of kidney transplantation contributing substantially to morbidity and mortality. Early diagnosis of the infection is essential to implement an effective antiviral therapy. This study is aimed at establishing the CMV antigenemia assay for the diagnosis of CMV infection, and for monitoring the effectiveness of ganciclovir therapy in kidney recipients in Kuwait. ***Methods:*** Leukocyte-enriched fractions of 215 blood specimens from 92 kidney recipients were processed for the detection of CMV-specific protein (pp65) by using a commercial kit (Incstar Corp., USA). Plasma fractions were also tested for the presence of CMV IgM antibodies, and selected samples (n = 15) were processed for virus isolation in MRC-5 diploid cell culture. ***Results:*** Regarding symptomatic (n = 35) and asymptomatic patients (n = 57) the assay showed an 86% positive and a 79% negative predictive value, compared to the 55% and 70% values, respectively, for CMV IgM antibodies. The number of positive cells was parallel with the severity of symptoms. Using a cutoff level of >5 antigen-positive leukocytes/50,000 cells, the assay reached 93% positive and 96% negative predictive values. There was a very good agreement between the assay and virus isolation. Ganciclovir therapy could be monitored effectively with the assay, and in 11 symptomatic patients treated with ganciclovir, a prompt decrease in the number of antigen-positive cells was noted. ***Conclusion:*** The CMV antigenemia assay is a valuable tool for the early diagnosis of symptomatic CMV infection and monitoring antiviral therapy in kidney transplant patients.

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Introduction

In transplant patients active cytomegalovirus (CMV) infection frequently occur and cause serious disease [1]. The incidence of clinical manifestation varies between 40 and 90% [2]. Antiviral therapy, when implemented early in a CMV infection, reduces the mortality of CMV disease substantially [3, 4]. In kidney and other transplant patients, it is essential to diagnose active CMV infection as early as possible. Rapid diagnostic tests are of practical value to differentiate CMV infection from other infections and to guide the use of antiviral therapy [5].

Isolation of infectious virus in cell culture is still considered the gold standard in the diagnosis of CMV infection [6]. However, the potential of virus isolation for the early diagnosis of CMV infection is limited by the long time requirement and the relatively low sensitivity of the method [7]. Polymerase chain reaction may have a strong impact on the diagnosis of CMV infection, but there is a need for further standardization and simplification [8].

Primary CMV infection may be diagnosed by serological methods. Although IgM detection is reliable to identify a recent CMV infection in healthy individuals, in immunocompromised patients, such as transplant recipients, CMV infection is produced in approximately 50–75% of the cases [6].

For the diagnosis of an active CMV infection, the detection of the CMV structural antigen (pp65) in polymorphonuclear leukocytes seems to be the best approach [9, 10]. The method now known as CMV antigenemia assay (AA) has been extremely useful for diagnosing active CMV infection in transplant patients [11–13].

In Kuwait, where several hundreds of transplant patients are under clinical surveillance, rapid and reliable laboratory diagnosis of CMV disease is essential. Therefore, we studied the usefulness of the AA for early diagnosis of CMV infection in kidney transplant patients.

Materials and Methods

Study Population. We enrolled 92 kidney transplant patients (35 females and 57 males), who received their transplants during 1996 and 1997. Their age spanned from 18 to 60 years, and 78% were between 30 and 45 years of age. All patients received an immunosuppressive regimen of cyclosporin A, azathioprine and steroids. One blood sample was collected from 38 patients, and two or more from 54 patients. From 15 of those 32 patients who developed any of the symptoms associated with CMV disease (unexplained fever, arthralgia, leukopenia, thrombocytopenia, elevated aminotransferase level, pneumonitis, hepatitis, retinitis, gastrointestinal ulceration), serial samples at different intervals were collected after transplantation and patients were monitored for 6–20 weeks. Eleven of these patients were treated with ganciclovir. Blood samples were collected in EDTA-containing tubes and transferred to the laboratory within 2–3 h. All the samples were processed for antigen detection on the same day. Plasma fractions of the samples were tested for the presence of CMV-IgM. Part of the separated cells were stored at –70°C for virus isolation.

CMV AA. From each patient 5 ml of blood was collected and processed immediately to isolate leukocytes by the use of dextran sedimentation method. Following incubation and centrifugation, the cell pellet was suspended in phosphate-buffered saline (PBS), erythrocytes were lysed with 0.8 m*M* ammonium-chloride, centrifuged, washed in PBS, counted and spotted onto glass slides (50,000 cells per spot). They were then dried and fixed in acetone bath, stained with Incstar CMV-vu Kit according to recommended procedures. The number of cells containing the CMV-specific pp65 antigen were counted with a light microscope (× 400).

Virus Isolation. Diploid fibroblast cell monolayers grown in LabTek 4-chamber slides were inoculated with polymorphonuclear blood leukocytes stored at –70°C. Before inoculation, cells were treated with 3 cycles of freezing and thawing. Two chambers were used for each sample. After inoculation cultures were incubated for 2 h at 37°C, fed with a medium and incubated further for 3–4 days in the presence of 5% CO_2. Then the cells were fixed in cold methanol, washed with PBS, incubated immediately with a

Table 1. Presence of CMV pp65 antigen in leukocytes of kidney recipients

AA	Patients	Samples	Patients	
			with symptoms	without symptoms
Positive	43 (47%)	79 (37%)	31 (89%)	12 (21%)
Negative	49 (53%)	136 (63%)	4 (11%)	45 (79%)
	92	215	35/92 (38%)	57/92 (62%)

monoclonal antibody to CMV early antigen (Biosoft, Paris, France) for 2 h. Finally, fluorescein-conjugated antimouse IgG was added to the cells and incubated for 1 h. Cells showing greenish fluorescence were detected by UV microscopy.

Serology. CMV-specific IgM antibodies (CMV-IgM) were detected with the use of conventional ELISA (Sorin, Biomedica), and/or immunofluorescence tests and confirmed with CMV-IgM blot assay (Genlabs Diagnostics, Singapore).

Statistics. For calculating the positive predictive value (PosPV) and the negative predictive value (NegPV) the following formulae were used: PosPV = (number of patients with positive test result and CMV-related disease/total patients with positive test result) × 100. NegPV = (number of patients with negative test result and without CMV-related symptoms/total patients with negative test result) × 100.

Results

Presence of CMV Antigen in Leukocytes. Among the 92 patients, 35 had symptomatic infection and 32 of them were positive for the pp65 antigen. Among the 57 asymptomatic patients, 12 patients had CMV antigen in their leukocytes (table 1). Accordingly the PosPV and NegPV of the assay are 86 and 79%, respectively. However, in 15 patients monitored for a longer period of time (6–20 weeks) by testing 3 or more consecutive samples from each patient, the presence of antigen correlated even more closely with the clinical condition. All of the 11 patients who had symptomatic infection during the follow-up period were positive by the AA.

Table 2. Correlation between AA and CMV-IgM test

AA	IgM test		Total
	positive	negative	
Positive	33 (42%)	46 (58%)	79
Negative	20 (15%)	116 (85%)	136
Total	53	162	215

Comparison of AA and IgM Detection. From the 92 patients 215 samples were collected and tested both with the AA and the IgM test. CMV-IgM could be detected only in 42% of the 79 antigen-positive samples. On the other hand, there was a relatively good agreement between the two tests regarding the 136 antigen-negative samples; 85% of them were negative for the presence of CMV-IgM (table 2).

When symptomatic and asymptomatic patients were compared (table 3), the result showed that IgM antibody could be detected in only 17 of the 35 symptomatic patients. The PosPV was 55% and the NegPV of the test (70%) approximated that of the AA (79%).

Table 3. Presence of pp65 antigen and CMV IgM antibodies in patients with and without clinical symptoms

	Patients			
	symptomatic		asymptomatic	
	positive	negative	positive	negative
AA	31/35 (89%)	4/35 (11%)	12/57 (21%)	45/57 (79%)
CMV-IgM	17/35 (49%)	14/35 (40%)	14/57 (25%)	43/57 (75%)

Table 4. Number of pp65 antigen-positive leukocytes in symptomatic and asymptomatic patients

Patients	Positivity scale				
	very low	low	high	very high	total
Symptomatic (n = 35)					
n	6	9	10	6	31
%	17.1	25.7	28.6	17.1	88.5
Asymptomatic (n = 57)					
n	10	2	0	0	12
%	17.5	3.5	–	–	21.0

Positivity scale: very low = 1–5 positive cells; low = 6–10 positive cells; high = 11–50 positive cells; very high = >50 positive cells.

Comparison of AA and Virus Isolation. In selected samples AA was compared to virus isolation. A strong agreement between the two methods was noted. Only one antigen-positive sample was negative in virus isolation.

Number of Antigen-Positive Leukocytes and Clinical Symptoms. The number of antigen-bearing cells varied extensively, from 0 to several hundred. For rating the positivity the following arbitrary scale has been formulated: 1–5 cells = very low positive; 6–10 cells = low positive; 11–50 cells = positive, and >50 = high positive. Grouping antigen-positive leukocytes detected in symptomatic and asymptomatic patients by this scale shows that detecting just a few cells (1–5) among the 50,000s screened did not give a definite indication of an ongoing CMV infection. On the other hand, the presence of more than 5 positive cells was strongly associated with clinical symptoms. With a cutoff of more than 5 cells as a positive test, the AA has a 93% PosPV and a 96% NegPV (table 4).

Positive AA and Ganciclovir Treatment. Eleven patients have received ganciclovir treatment for various periods of time (from 2 to 8 weeks). One patient became antigen-positive 8 weeks after transplantation and was symptomatic. Ganciclovir therapy was started and continued for 2 weeks. When the patient was tested 4 and 8 weeks later he was asymptomatic and negative in the AA. Four patients followed the same pattern becoming antigen-negative and asymptomatic after the antiviral treatment. Four other patients had a somewhat different course. At the time when clinical symptoms were present, a high number of antigen-positive leukocytes could be detected. Their number reduced gradually but positive cells were still present when patients became asymptomatic. One patient who had a multiple, concomitant disease process including pneumococcal meningitis responded to ganciclovir treatment, and the initially high number of positive cells (175) was reduced to 4. Another patient who responded to ganciclovir treatment (antigen-positive cells could not

be detected at week 12 after transplantation) presented with central nervous system lymphoma and became critically ill. There were 4 patients with symptoms who did not receive antiviral treatment. Two of them were very low positive (1–2 cells) in the AA and they became asymptomatic spontaneously. The other 2 patients had an increase in the number of antigen-positive cells and presented with CMV-compatible symptoms. Unfortunately, their follow-up could not be continued further because they left the country.

Discussion

Detection of the CMV-related pp65 antigen in peripheral leukocytes of transplant patients has become an increasingly important assay for the diagnosis of CMV infection [14, 15]. The CMV AA has substantial advantages: it gives rapid results (available within 5–6 h), correlates exceptionally well with symptomatic CMV infections, and can be used for monitoring the effectiveness of antiviral therapy [16]. The assay is relatively easy to perform and inexpensive when compared to either cell culture or PCR methodologies [17].

In this study, the diagnostic significance of the AA was determined on 92 kidney transplant patients with and without symptomatic infections. Among the 35 symptomatic patients there were 4 AA-negative patients. All of them presented only with one clinical symptom (unexplained fever) which may not have been related to CMV infection. The 31 patients who had more than one CMV-related symptom were positive by the AA. Among the 57 asymptomatic patients, 12 had the antigen in their leukocytes, however, the number of antigen-positive cells never exceeded 10. If we used a cutoff value >10 positive cells none of the asymptomatic patients would fall into the positive range. Indeed quantification seems possible [18, 19] and it is important in relation to patient management [10]. The higher the number of antigen-positive cells the closer is the association with more severe symptomatic CMV infections.

Although in this study only 15 selected samples were tested for both the AA and virus isolation, the result showed a good agreement between the two tests. There was only 1 AA-positive case with negative virus isolation but the number of antigen-positive cells was very low (2 cells only).

The scale upon which the positivity of the AA is determined may differ from one laboratory to the other, however, it does not jeopardize the value of the assay. Since several modifications of the assay are available [20], it would not be reasonable to set a unified rating scale.

A comparison between AA and CMV-IgM showed that only 49% of symptomatic cases had CMV IgM antibodies, which means that the PosPV of the IgM test is much lower (55%) than that of the AA (86%). It may be due to several factors like antibody status before transplantation, source of grafts, immunological compatibility, immunosuppressive therapy etc., which influence the production of IgM in organ transplant patients [18]. It is of interest that the 4 patients with the highest number of antigen-positive cells did not produce detectable CMV IgM antibodies.

The assay has an obvious impact on monitoring ganciclovir therapy. In all the 11 patients treated with the drug, the number of CMV antigen-positive cells decreased promptly as the clinical conditions of the patients improved. The 4 patients without treatment had a relatively low number of AA-positive cells. Nevertheless, 2 patients were symptomatic when they had more than 5 positive cells. Unfortunately their follow-up could not be continued.

In summary we conclude that the CMV AA is a valuable laboratory method for diagnosing CMV infections and monitoring anti-CMV therapy in kidney transplant patients.

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