CMV-, EBV-, and HHV-6-DNAemia after liver transplantation

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Loginov R, Höckerstedt K, Lautenschlager I. Detection of CMV-DNA in peripheral blood leukocytes of liver transplant patients after ganciclovir treatment. Archives of Virology 2003; 148(7):1269-74.

II Härmä M, Loginov R, Piiparinen H, Halme L, Höckerstedt K, Lautenschlager I. HHV-6-DNAemia related to CMV-DNAemia after liver transplantation. Transplantion Proceedings 2005; 37(2):1230-32.

III Loginov R, Aalto S, Piiparinen H, Halme L, Arola J, Hedman K, Höckerstedt K, Lautenschlager I. Monitoring of EBV-DNAemia by quantitative real-time PCR after adult liver transplantation. Journal of Clinical Virology 2006; 37(2):104-108.

IV Loginov R, Härmä M, Halme L, Höckerstedt K, Lautenschlager I. HHV-6 DNA in peripheral blood mononuclear cells after liver transplantation. Journal of Clinical Virology 2006; 37 Suppl 1: S76-S81.

ABBREVIATIONS

CAN	Chronic allograft nephropathy
CMV	Cytomegalovirus
DGTP	Deoxyguanosine triphosphate
DR	Direct repeat
EBV	Epstein-Barr virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
HSCT	Haematopoietic stem cell transplantation
HSV-1	Herpes simplex 1
HSV-2	Herpes simplex 2
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
IR	Internal repeat
ES	Exanthema subitum
KSHV	Kaposi`s sarcoma-associated herpesvirus
NF-κB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cells
PTLD	Post-transplant lymphoproliferative disorders
RSV	Respiratory syncytial virus
TNF-α	Tumor necrosis factor-alpha
TR	Terminal repeat
US	Unique short region of genome
UL	Unique long region of genome
VCAM-1	Vascular cell adhesion molecule-1
VZV	Varicella Zoster virus
VBDS	Vanishing bile duct syndrome

ABSTRACT

Viral infections caused by herpesviruses are common complications after liver transplantation and they are associated with substantial morbidity and even mortality. Primary infections of these viruses are rare in adult transplant patients, due to the high sero-prevalence already in early childhood. An important characteristic of human herpesviruses is their ability to remain in a latent state in a host after primary infection and reactivate later. CMV is clinically the most important herpesvirus occurring after liver transplantation. Less is known about the significance of another betaherpesvirus, HHV-6. EBV is believed to play a major role in the development of post-transplant lymphoproliferative disorders (PTLD).

The aim of this study was to investigate the CMV-, EBV- and HHV-6-DNAemia after liver transplantation, by frequent monitoring of adult liver transplant patients who had been transplanted at the Transplantation and Liver Surgery Clinic, Department of Surgery, Helsinki University Hospital between the years 1999-2005. The clinical material comprised of blood specimens obtained weekly during the patients` post-operative hospitalization and thereafter according to our clinical protocol, and in case of clinical symptoms. The presence of CMV, EBV and HHV-6 DNA were demonstrated by *in situ* hybridization assays and by real-time PCR methods. CMV and HHV-6 antigens were demonstrated by antigenemia assays and compared to the viral DNAemia. The response to antiviral therapy was also investigated.

The expression of CMV-DNA in peripheral blood leukocytes was monitored and compared with CMVpp65-antigenemia in liver transplant patients receiving ganciclovir treatment. CMV-DNA expression preceded pp65-antigenemia. All patients responded to ganciclovir and CMV antigenemia subsided. However, most of the treated patients demonstrated persistence of CMV-DNA for up to six months and recurrences appeared in many cases. Continuous CMV DNA expression of peripheral blood leukocytes showed that the virus is not eliminated by ganciclovir and recurrences can be expected during several months after liver transplantation.

HHV-6-DNAemia was investigated in relation to CMV-DNAemia by quantitative PCR. HHV-6-DNAemia/antigenemia was common and usually associated with CMV in liver transplant recipients. Concurrently with CMV, HHV-6-DNAemia and antigenemia were detected in most patients. However, the HHV-6 viral loads were low in all cases. All CMV infections were successfully treated with ganciclovir and the CMV-DNAemia/antigenemia disappeared. In ganciclovir treated patients, also HHV-6-DNAemia/antigenemia responded to the treatment, but more slowly and less clearly.

HHV-6 DNA was demonstrated by *in situ* hybridization from peripheral blood mononuclear cells in parallel with HHV-6 antigenemia. The number of HHV-6 DNA positive cells was quantified to determine the viral load. The HHV-6 DNA expression correlated well with HHV-6 antigenemia. Antiviral treatment significantly decreased the number of HHV-6 DNA positive cells demonstrating the response to ganciclovir treatment. These results suggest that the semi-quantitative HHV-6-DNAemia test may be used as an alternative tool in the monitoring of transplant recipients.

Clinically silent EBV reactivations with low viral loads were relatively common after liver transplantation, occurring in 13% of patients. These EBV-DNAemias usually appeared within the first 3 months after liver transplantation together with betaherpesviruses (CMV, HHV-6, HHV-7), and subsided within a few weeks. One patient developed PTLD with high viral loads. This patient first presented low-level EBV DNA in the early post-transplant period, but later developed PTLD with high-level EBV-DNAemia. These results indicate that frequent monitoring of EBV viral loads can be useful to detect liver transplant patients at risk of developing PTLD.

The findings presented in this thesis provide new information of CMV, EBV and HHV-6 reactivations and their clinical significance after liver transplantation in adult patients and the response of these viruses to the treatment.

REVIEW OF THE LITTERATURE

1. VIRAL INFECTIONS IN LIVER TRANSPLANTATION

Medical and surgical advances over the last decades have made a reality of long –term survival after liver transplantation. However, after transplantation lifelong immunosuppressive medication is necessary. Therapies used to prevent rejection after liver transplantation suppress the immune system and expose the recipient to a high risk of viral, bacterial and fungal infections (Paya and Razonable, 2003). More than two-thirds of liver transplant patients have infection in the first post-transplant year (Blair and Kusne, 2005). Bacterial infection is one of the most frequent and serious complications after liver transplantation and the majority of bacterial infections occur within two months post-transplantation. Fungal infections are also demonstrated after transplantation, with *Candida* spp. and *Aspergillus* spp. being the most common causes of infection (Tollemar, 2003).

Recurrence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection after transplantation are common complications in liver transplant recipients worldwide (Wright and Berenguer, 2003). The majority of liver transplants performed globally are for chronic viral hepatitis caused by HBV and HCV and disease recurrence after liver transplantation due to these viruses accounts for a significant proportion of morbidity, mortality and cost (Berenguer, 2002, Paya, 2001, Samuel et al., 1993). However, hepatitis B and C viruses are only a minor cause for liver transplantations here in Finland.

Adenovirus may cause infection in pediatric liver transplant recipient, but is less common in adult recipients (Kusne and Blair, 2006). The virus may cause colitis, hepatitis, pneumonitis, hemorrhagic cystitis and encephalitis in transplant recipients. Also other community respiratory viruses, such as respiratory syncytial virus (RSV), influenza viruses, parainfluenza viruses, rhinoviruses, enteroviruses and coronaviruses may infect transplant recipients and cause them numerous illnesses with various types of syndromes from self-limited upper respiratory tract illnesses to serious pneumonias (Englund and

Whimbley, 2003). Norovirus infections (Mattner et al., 2005) and rotavirus infections (Stelzmueller et al., 2006) may also complicate post-transplant period.

Viral infections caused by herpesviruses are common complications after liver transplantation and they are associated with substantial morbidity and even mortality (Paya and Razonable, 2003). Thus, it is important to diagnose and treat these infections early. The human herpesviruses that may cause infection after liver transplantation are herpesvirus-1 (HSV-1) and -2 (HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus-6 (HHV-6), and human herpesvirus-7 (HHV-7) (Kusne and Blair, 2006, Singh 2000). The primary infections of these herpesviruses are rare in adult liver transplant patients due to the high sero-prevalence already in early childhood. Another gamma-herpesvirus, HHV-8 or Kaposi`s sarcoma –associated herpesvirus (KSHV), may in rare occasions infect transplant recipients (Singh, 2000). The main character of human herpesviruses is the ability to remain in a latent state in a host after primary infection and reactivate later.

CMV is one of the most important herpesvirus after liver transplantation and may occur in the absence of symptoms, or it may result in symptomatic infection with mild to life-threatening symptoms. The role of CMV in post-transplantation clinical illness and outcomes are well characterized (Paya and Razonable, 2003). In the management of CMV infection of transplant recipients, major advances have been achieved through the development of new, rapid diagnostic techniques for monitoring patient`s viral loads and through the use of anti-viral agents. Two other betaherpesviruses, HHV-6 and HHV-7, have in recent years been associated with clinical syndromes, although their role in solid-organ transplant recipients is not well defined (Singh, 2003).

EBV is a gamma-herpesvirus, which persists in a latent state. Most primary infections occur in pediatric transplant recipients, whereas in adult patients reactivations are seen. Infection with EBV is believed to play major role in the development of post-transplant lymphoproliferative disorders (PTLD) (Preiksaitis and Keay, 2001). Although uncommon with a low incidence in solid-organ transplantations, the EBV-related PTLD is an aggressive disease, with high mortality rates of up to 80% (Paya et al., 1999).

2. THE HUMAN HERPESVIRUSES

The family *herpesviridae* consists of eight members: herpes simplex 1 and 2, varicella zoster, Epstein-Barr virus, cytomegalovirus and human herpesvirus 6, 7 and 8 (Zhou et al., 2006). The herpesvirus family is divided into three subfamilies, designated alpha, beta, and gamma. HSV-1, HSV-2, and VZV belong to the alphaherpesviruses, whereas CMV, HHV-6 and -7 are betaherpesviruses and Epstein-Barr (EBV) virus and HHV-8 are gammaherpesviruses (Zhou et al., 2006). HSV-1 and HSV-2 are neurotropic and cause fever blisters, genital sores and occasionally central nervous infections. Primary varizella-zoster virus infection causes chickenpox (varicella), usually in children. The recurrent form of VZV is herpes zoster in immunosuppressed and elderly persons. In immunosuppressed individuals VZV may also cause central nervous infection or in rare cases even visceral VZV disease without cutaneous involvement (Grant et al., 2002). EBV is the cause of the majority of mononucleosis cases. The EBV virus is also associated with post-transplant lymphoproliferative disorders (PTLD) and other malignancies, such as naso-pharyngeal carcinoma and Burkitt`s lymphoma. HHV-8 is etiologically linked to Kaposi's sarcoma and other malignancies. CMV primary infections of immunocompetent individuals are usually asymptomatic but might also cause mononucleosis-like illness and in rare cases even hepatitis. Congenital infection caused by CMV is a major cause of hearing loss and mental retardation. Moreover, reactivation of the virus is important in immunocompromised patients, such as transplant recipients and AIDS patients. HHV-6 is the causative agent of *exanthema subitum* (ES), an early childhood disease characterized by high fever and a mild skin rash, which are occasionally complicated by seizures or encephalitis (Yamanishi et al., 1988). HHV-6 may also reactivate during immunosuppression. The clinical manifestations of HHV-7 are less clear, but have been associated with some cases of *exanthema subitum* (Tanaka et al., 1994, Toriqoe et al., 1995, Ueda et al., 1994), pityriasis rosea (Drago et al., 1997), neurological symptoms (Caserta et al., 1998, Torigoe et al., 1996) and hepatitis (Hashida et al., 1995).

The main characteristics of herpesviruses are their ability to remain in a latent or persistent state in their host after primary infection and reactivate under

immunosuppression. Reactivation and infection with human herpesviruses remain one of the most common infectious complications after transplantation (Fishman and Rubin, 1998).

3. HUMAN CYTOMEGALOVIRUS (CMV)

3.1. CMV virus

3.1.1 Structure and genome

Human cytomegalovirus (CMV) is the largest known human herpesvirus with a genome of about 230kb and it belongs to the beta-herpesvirus group together with HHV-6 and 7 (Landolfo et al., 2003). The virion consists of an icosahedral nucleocapsid containing a double-stranded linear DNA genome surrounded by a proteinaceous layer, defined as the tegument or matrix, which is then enclosed by a lipid bilayer, containing viral glycoproteins (Mocarski and Courcelle, 2001). The mature virion particle is 150-200nm in diameter. The genome of CMV contains unique long (UL), unique short (US), and repeat regions and it encodes over 200 proteins. Each long and short region can be oriented in either direction so that four genome isomers are produced in viral progeny. Direct repeat sequences at the genome termini and inverted repeat elements at the UL-US junction mediate the inversion of UL and US regions. Four proteins constitute the capsid: pUL46, pUL48.5, the minor capsid protein (mCP, UL85) and major capsid protein (MCP, UL86). The phospholipid envelope contains six virus encoded glycoproteins (gB, gN, gO, gH, gM, gL) and these play essential roles in viral entry into host cells, cell-to-cell spread and virion maturation (Britt and Mach, 1996). There are 20-25 structural virion proteins, which are located in the amorphous layer between the nucleocapsid and the envelope (Baldick and Schenk, 1996). Most of these tegument proteins are phosphorylated and they are also highly immunogenic. The most abundant are ppUL32 (pp150) and ppUL83 (pp65), of which the latter is the target antigen in antigenemia assays for rapid diagnosis of CMV infections.

3.1.2 Replication

The cellular receptor of CMV is unknown, but due to the ability of CMV to recognize and productively enter such a wide range of cells suggest that the receptor is widely distributed (Mocarski and Courcelle, 2001). The epidermal growth factor receptor (EGFR) and cellular integrins have recently been suggested to function as entry receptors for CMV (Feire et al, 2004, Wang et al., 2003). Viral entry is the result of a cascade of interactions between viral and cellular proteins and it ends in fusion of the virion envelope with the plasma membrane (Fig.1). After that, the virus capsid is transported to the nucleus and the viral DNA is released into the nucleus.

During productive replication the viral genes are expressed in a temporally coordinated and regulated cascade of transcriptional events that lead to synthesis of immediate-early (IE), early (E), and late (L) proteins of virus (Mocarski and Courcelle, 2001). In general, the IE proteins are activators of other genes. They have an important role in controlling cellular and viral gene expression, especially of E gene expression. E genes encode mostly non-structural proteins, which are involved in the replication of viral DNA and in the induction of L gene expression. L proteins are the virus structural proteins and they have a role in viral assembly and morphogenesis of the virion. DNA replication, formation of viral capsids and DNA packaging occur in the nucleus, whereas the maturation takes place in the cytoplasm before the mature virus particles leave the cell via exocytosis. The whole replication cycle of human cytomegalovirus takes approximately 48-72 hours.

3.1.3 Tropism

During natural infection, it is shown that CMV replicates in many kinds of cells, including epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, mesenchymal cells, hepatocytes, monocytes/macrophages and lymphocytes (Kahl et al., 2000, Plachter et al., 1996, Sinzger and Jahn., 1996, Sinzger et al., 1996). CMV may also infect several types of tissues, including most of the parenchymal organs, salivary glands, gastrointestinal and genitourinary tract (Pass, 2001)

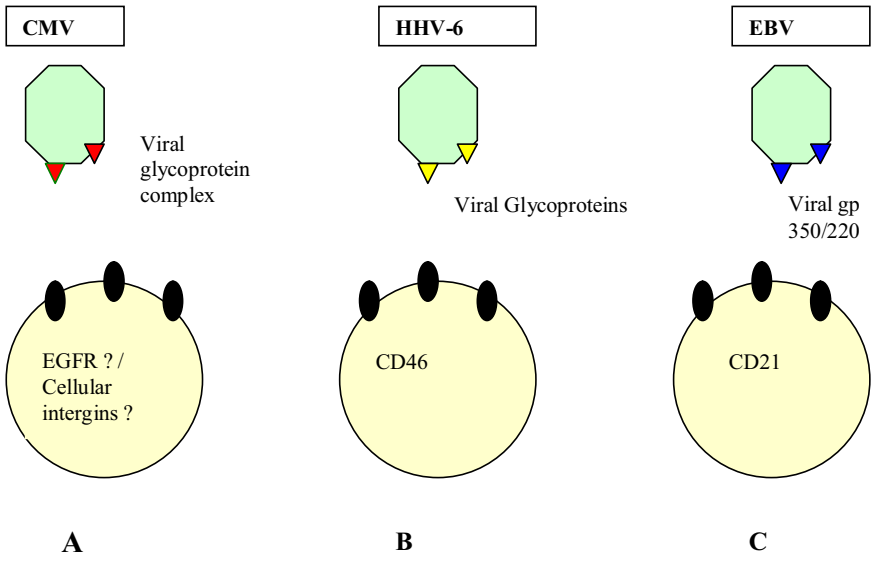


Figure 1 The viral entry on the host cell is a cascade of interactions between viral and cellular proteins and it ends in fusion of the virion envelope with the plasma membrane. **A)** The epidermal growth factor receptor (EGFR) and cellular integrins has been suggested to function as entry receptors for CMV. These interact with viral glycoprotein complex. **B)** HHV-6 enters the cell through interaction with CD46. Glycoprotein complex (the glycoproteins H, L and Q) of HHV-6 acts as the viral ligand for human CD46. **C)** Infection of EBV is initiated by the binding of the major outer envelope glycoprotein gp 350/220 of EBV with the cellular complement receptor type 2 (CR2), also known as CD21.

3.1.4 Epidemiology and transmission

CMV is a widespread pathogen causing generally asymptomatic, persistent infection during the first two decades of life (Pass, 2001). Primary infection is usually mild and subclinical but it may cause mononucleosis-like illness. Infection is lifelong and after the primary infection the latent CMV may reactivate and produce infectious virions that are shed in saliva, urine, blood, tears, semen and breast milk. The seroprevalence varies between 40-100% in different countries and socioeconomic groups, being 70-80% in Finland and the Scandinavian countries (Ho et al., 1990, Pass, 2001). CMV infection can be transmitted both vertically from mother to children and horizontally from person to

another. One of the clinical manifestations of primary infection is congenital CMV infection (Landolfo et al., 2003). Maternal genital secretions, breast milk or saliva are the main perinatal/post-natal route of infection. In normal transmission of the virus in children and adolescents, close contact is required, and the route is thought to be saliva and later sexual contact. In immunocompromised patients, such as patients with an immunosuppressive treatment or AIDS, CMV infection may lead to life-threatening disease. Very important transmission routes in these patients are blood and transplanted organs (Paya and Razonable, 2003). Recently, the use of leukocyte-depleted blood products has decreased the risk of CMV transmission via blood (Boechk et al., 2004).

3.1.5 Latency

Like other herpesviruses CMV can establish latency. Virus may persist in specific sites in the host after primary infection, but without any detectable production of infectious virus (Sinclair and Sissons, 2006). Sporadic reactivation events may occur, but they are generally well-controlled by cell-mediated immunosurveillance. Occasionally CMV activates from its latent state and infectious virions appear in the saliva and/or urine. Reactivations of CMV in immunocompetent individuals are entirely asymptomatic, but form an important route for CMV to spread horizontally and vertically. However, when these reactivations occur in immunocompromised patients, CMV replication can become uncontrolled and may cause severe life-threatening infections. The sites of latency are blood leukocytes, mainly mononuclear cells, but viral DNA has also been detected in the early bone marrow haematopoietic progenitors, epithelial cells and endothelial cells (Hendrix et al., 1990, Kondo et al., 1994, Markovic-Lipkovski et al., 1992, Melnick et al., 1983, Taylor-Wiedeman et al., 1991, 1993, Sindre et al., 1996). Reactivation of a latent virus by allogeneic stimulation has been demonstrated (Soderberg-Naucler et al., 1997). The bone marrow acts as a reservoir of latent CMV, which then seeds latent virus into the peripheral blood via monocytes. Differentiation of monocytes to tissue macrophages seems to lead to virus reactivation and productive infection (Sinclair and Sissons, 2006, Söderberg-Naucler et al., 2001, Taylor-Wiedeman et al., 1994).

The reactivation of CMV from latency is the critical first step in the pathogenesis of CMV infection. The key mediator for the reactivation of CMV from latency is TNF-alpha, which activate protein kinase C and NFκB, a promoter of the immediate early (IE) gene of CMV initiating replication (Prosch et al., 1995, Stein et al., 1993). This observation is important, as it explains why CMV infection and disease are associated with several processes (e.g. sepsis, other viral infections and allograft rejection) (Rubin, 2001).

3.2 CMV after transplantation

CMV infection and disease are important causes of morbidity and even mortality among transplant patients. The definitions of CMV infection and disease in transplant recipients have recently been updated (Ljungman et al., 2002). "CMV infection" is defined as isolation of the CMV virus or detection of viral proteins or nucleic acid in any body fluid or tissue specimen (Ljungman et al., 2002). The minimum requirements for definition of "CMV disease" are fever (>38ºC, for at least 2 days within a 4-day-period), neutropenia or thrombocytopenia, and the detection of CMV in blood. In an end-organ disease, e.g pneumonia, hepatitis, gastrointestinal disease, retinitis, nephritis, cystitis, myocarditis, pancreatitis or central nervous disease, the CMV virus needs to be detected in the particular organ. CMV pneumonia remains a life-threatening syndrome, which is usually complicated by other pathogens, such as *Pneumocystis*, *Aspergillus* and other fungal co-pathogens (Fishman and Rubin 1998). In hematopoietic stem cell transplantation (HSCT) patients, the most frequent clinical manifestations of CMV are fever, pneumonitis and gastrointestinal disease (Boeckh and Ljungman, 2003). In solid organ transplant patients, CMV can infect various organs such as lung, liver, intestines, kidney and heart, and may also affect the transplanted organ (Paya and Razonable, 2003).

In addition to direct effects of CMV on the host, there are also indirect effects of the virus (Rubin, 2001). These are an association of CMV with acute graft rejection (Pouteil-Noble et al., 1993, Reinke et al., 1994) and chronic graft rejection, including accelerated transplant vasculopathy in heart transplant recipients (Grattan et al., 1989, Koskinen et

al., 1993, Valantine et al., 1999), vanishing bile duct syndrome (VBDS) in liver transplant recipients (Arnold et al., 1992, Evans et al., 1999, Lautenschlager et al., 1997), chronic allograft nephropathy (CAN) in kidney transplant recipients (Helanterä et al., 2003, Humar et al., 1999, Tong et al., 2002) and even bronchiolitis obliterans in lung recipients (Kroshus et al., 1997). CMV is also associated with bacterial and fungal infections (Fishman and Rubin, 1998, George et al. 1997). CMV could interact with other viruses and may accelerate hepatitis C virus pathogenesis (Burak et al., 2002, Razonable et al., 2002c). On the other hand, HHV-6 and CMV infection and viral load were not associated with increased overall rates of HCV recurrence or HCV viral load after liver transplantation but may be associated with more severe forms of recurrence (Humar et al., 2002b).

CMV infection occurs in the majority of solid organ transplant recipients, during the first 3 post-transplant months, when the immunosuppression is most intense (Paya and Razonable, 2003). The incidence of CMV disease varies from 8% to 50%, depending on the type of transplanted organ (Paya and Razonable, 2003). CMV sero-negative recipients, who receive an organ from a sero-positive donor, may develop primary infection. However, the adult recipients are mostly CMV sero-positive, and the post-transplant infections are reactivations of the latent virus of the recipient, but may alternatively be reinfections caused by a latent virus of donor origin. Primary infections have been associated with more severe forms of disease than reactivations or reinfections (Fishman and Rubin 1998). The risk factors for CMV disease in transplant recipients include CMV sero-positive donor / CMV sero-negative recipient (D^+/R^-) and the intensity of immunosuppressive therapy. Also the type of transplantation has an effect on the occurrence of CMV disease: lung and heart-lung transplant recipients are at highest risk for CMV disease, liver or heart transplant recipients have an intermediate risk, and kidney transplant recipients are at the least risk (Paya and Razonable, 2003).

In liver transplant recipients, CMV infection is common and the incidence of CMV disease has been described to be up to 29% (Paya and Razonable, 2003). CMV hepatitis is the most common complication of CMV infection after liver transplantation with an incidence of 2% to 11% (Lautenschlager et al., 2006, Paya and Razonable, 2003,

Seehofer et al., 2002). However, CMV hepatitis seems to have no influence on the long-term outcome of patients, but biliary complications were found to be common (Halme et al., 2003, Lautenschlager et al., 2006). Due to modern monitoring and effective antiviral treatment, the incidence and severity of CMV disease is decreasing (Seehofer et al., 2004). CMV has also been associated in liver transplant recipients with other betaherpes viruses, such as HHV-6 and HHV-7 (Humar et al., 2002a, Lautenschlager et al., 2002b, Mendez et al., 2001).

3.3 Diagnosis and therapy

Because CMV causes a significant disease to transplant patients, strategies aimed at CMV prevention have been developed. These strategies include "prophylaxis", "pre-emptive therapy" and treatment of established CMV disease (Ljungman, 2002). In the prophylaxis, the patients at risk of disease based upon the pre-transplant CMV serostatus of recipient and donor are treated after transplantation. In the pre-emptive therapy, antivirals are given in situations, where there is laboratory evidence of viral replication by viral culture, pp65 antigenemia, or detection of CMV DNA. Treatment is given following established CMV disease in transplant recipients.

The diagnostic methods include serology, viral culture, antigen detection and detection of nucleic acids. Several specific definitions for CMV in blood are recommended: viremia is defined as the isolation of CMV by culture from blood, antigenemia as the detection of CMV pp65 in blood leukocytes, and DNAemia as the detection of DNA in samples of plasma, whole blood, isolated peripheral blood leukocytes or buffy-coat specimens (Ljungman et al., 2002). Serology is most useful in the detection of past exposure to CMV in both donor and recipient. As the majority of transplanted recipients are sero-positive (IgG) for CMV, the clinical usefulness of serology in diagnosing CMV infection is poor (Razonable et al., 2002b). The diagnosis of CMV infection in organ transplant recipients is based on detection of virus in blood, urine, biopsies or bronchoalveolar lavage fluid. The infectious virus can be isolated by conventional or by shell-vial culture. The detection of CMV in cell cultures from the blood is not an ideal marker to guide

antiviral treatment due its low sensitivity (Boeckh and Boivin, 1998). However, viral culture still provides a sensitive method to detect an active virus in various body fluids, such as urine and BAL.

The diagnosis of CMV infection and disease has evolved considerably in recent years with the introduction of quantitative tests. Nowadays, the diagnosis of CMV infection in transplanted patients is mainly based on frequent monitoring of peripheral blood by the pp65 antigenemia assay or by quantitative PCR (Boeckh and Boivin, 1998). The antigenemia assay is a rapid quantitative method to detect CMV antigens in polymorphonuclear leukocytes with monoclonal antibodies, which are specific for the CMV lower matrix protein pp65 (UL83) (The et al., 1995). Quantitative results are expressed as the number of CMV positive cells per blood leukocytes.

The recent availability of automated PCR instruments has improved the diagnosis of CMV infection. Various commercial methods have been developed to detect CMV DNA viral load, such as The COBAS AMPLICOR CMV MONITOR assay (Roche Diagnostics), which amplifies a segment of the viral DNA polymerase gene UL54 within a turnaround time of approximately 4 hours (Sia et al., 2000). This assay has shown a good correlation to pp65 antigenemia test in the diagnosis and monitoring of transplant patients (Humar et al., 1999, Piiparinen et al., 2001, Rollag et al., 2002, Tong et al., 2000). TagMan based quantitative real-time PCR for the detection of CMV-DNA in plasma specimens has also been used in transplant recipients and has been found a good choice for the diagnosis and monitoring of CMV in solid organ transplant recipients (Meyer-Koenig et al., 2004, Piiparinen et al., 2004). There are several possible techniques that can be used for the detection of CMV in tissue biopsies. These include the detection of viral antigens in the tissue specimens by immunostaining using monoclonal antibodies and the detection of viral DNA by in situ-hybridization techniques (Ljungman et al., 2002).

The currently available antivirals for the treatment of CMV disease after transplantation are ganciclovir, foscarnet, and cidofovir (Paya and Razonable, 2003). Ganciclovir is a choice for the treatment of established CMV disease in solid organ transplant patients,

given intravenously for at least two weeks. Ganciclovir is a nucleoside analogue, which in its active triphosphorylate form inhibits the viral DNA polymerase and competes with deoxyguanosine triphosphate (DGTP) to act as a terminator of biosynthesis for the viral DNA. This phosphorylation in CMV infected cells is mediated by the CMV UL97 protein (Littler et al., 1992). This protein is a phosphotransferase that phosphorylates ganciclovir to ganciclovir monophosphate in infected cells (Noble and Faulds, 1998, De Clerq 2004, Biron 2006). Two subsequent rounds of phosphorylation by cellular kinases produce ganciclovir triphosphate. Resistance to ganciclovir arises from mutations in the UL97 gene. Resistance may also arise from mutations in the viral DNA polymerase gene (UL54). However, ganciclovir resistance in transplant patients is a rare condition. The side-effects of ganciclovir include leukopenia and thrombocytopenia.

Foscarnet is a pyrophosphonate analogue, which interferes with the binding of the pyrophosphate to its binding site of the viral DNA polymerase and it is used when ganciclovir is contraindicated. The major dose-limiting toxicity of foscarnet is renal impairment. Resistance to foscarnet arises from the point mutations in the UL54 gene, and cross-resistance has been observed between ganciclovir and foscarnet. Cidofovir is a nucleoside analogue, which may also be used, but experience with this antiviral in solid organ patients is very limited (Biron 2006). In addition, CMV immunoglobulin may be used in lung transplant recipients for the treatment of CMV disease together with ganciclovir (Weill et al., 2003).

In prophylaxis, ganciclovir is administered before active CMV infection occurs, immediately after the transplantation, usually to the patients with a high risk of developing CMV disease, especially those D+/R- patients. The practice and guidelines of CMV prophylaxis varies widely depending on the type of transplantation and on the transplant center. Ganciclovir is a widely used and effective drug for CMV prophylaxis in solid organ transplant recipients. Previously, ganciclovir was administered intravenously, thereafter with the availability of the oral ganciclovir still favored CMV prophylaxis, although the bioavailability of oral ganciclovir is lower than intravenous ganciclovir (Gane e al., 1997, Paya et al., 2002, Winston and Busuttil, 2004). Valganciclovir, a valine ester of ganciclovir, was been developed more recently to overcome the limitations of

oral ganciclovir and it gives the same plasma ganciclovir exposures to those achieved with i.v. ganciclovir (Peskovitch et al., 2000). Valganciclovir prophylaxis in solid organ transplant patients has been found clinically as effective as oral ganciclovir with a comparable safety profile and the absence of ganciclovir resistance (Hodson et al., 2005, Paya et al., 2004,). Valganciclovir has also recently been used in the treatment of CMV infection and CMV disease in organ transplant recipients (Babel et al., 2004, Humar et al., 2005).

In preemptive therapy, antiviral treatment is initiated when CMV is detected in the blood using sensitive quantitative virological methods, such as PCR or tests for viral antigen. Preemptive therapy is based on frequent monitoring of the viral load in the peripheral blood of the recipient. It has been shown that peak viral loads correlate with CMV disease (Cope et al., 1997a, b, Gor et al., 1998), and that the rate of increase in viral load can identify recipients at risk of developing CMV disease (Emery et al., 2000). The advantages of the pre-emptive therapy include reduction in the number of patients exposed to antivirals, lowering the risk of drug resistance and maximizing the cost-benefit ratio (Landolfo et al., 2003). The effectiveness of pre-emptive ganciclovir therapy has also been shown in randomized, placebo-controlled trials (Paya et al., 2002, Strippoli et al., 2006). Recently, valganciclovir has also been used pre-emptively (Paya et al., 2004, Singh et al., 2005).

4. HUMAN HERPESVIRUS 6 (HHV-6)

4.1. HHV-6 virus

Human herpesvirus 6 (HHV-6) was first isolated from the peripheral blood of immunocompromised patients (Salahuddin et al., 1986). HHV-6 is a member of the β-herpesvirus group and closely related to cytomegalovirus (CMV) (Efstathiou et al., 1988). Two major subgroups of HHV-6 have been identified, variants A and B, of which variant B is the most common (Dewhurst et al., 1993). These two variants differ in their genetic, antigenic and growth properties (Ablashi et al., 1991, Chandran et al., 1992) .

4.1.1 Genome

HHV-6 genome is a linear, double-stranded DNA molecule, 160 to 162kb in size, flanked by terminal direct repeats (DR_{LEFT} and DR_{RIGHT}) of 8 to 9kb (De Bolle et al., 2005). The unique long (UL) region is interrupted by three intermediate repeats, R1,R2 and R3, in the immediate-early A (IE-A) region. The genomic organization of the unique region shares similarities with unique long (UL) region of human cytomegalovirus and it is also colinear with that of HHV-7. The genes in UL are termed U1 to U100 and open reading frames (ORFs) within the direct repeats are designated DR1 to DR7. Genes coding for structural virion components or enzymes required for nucleotide metabolism and DNA replication, are clustered into seven gene blocks. These are also conserved among all the herpesviruses. The HHV-6B genome contains 119 open reading frames encoded by 97 genes, nine of which are absent in HHV-6A (Domingues et al., 1999). The overall nucleotide sequence identity between HHV-6A and B variants is 90%. In the IE-region of the genome the highest degree of divergence between variants A and B is found (Chou and Marousek, 1994).

4.1.2 Replication

Both the A and B variants of HHV-6 enter the cell through interaction with CD46, which is a ubiquitous type 1 glycoprotein expressed on the surface of all nucleated human cells (Santoro et al., 1999). CD46 is a member of a family of glycoproteins acting as regulators of complement activation. It has been demonstrated that the gH-gL-gQ complex of HHV-6A (the glycoproteins H, L and Q being encode by the HHV-6 genes U48, U82 and U100), acts as the viral ligand for human CD46 (Mori et al., 2003). HHV-6A gB was found to be essential for virion penetration into the cell (Takeda et al., 1996). After binding to its receptor and gH-gL-gQ mediated fusion of the viral envelope (Fig.1), the incoming nucleocapsid is transported through the cytoplasm to the nuclear pore complexes, where the viral DNA genome is then released into the nucleoplasm (De Bolle, et al, 2005, Zhou et al., 2006). In the nucleus, the virus uses the cellular transcription and translation machinery and the viral gene expression occurs in a

temporally ordered cascade. Immediate early (IE) gene expression is activated first followed by early (E) and late (L) gene expression. The IE proteins are transactivators of gene expression and they regulate the expression of other genes. The early proteins are involved in the replication of the viral DNA, whereas late proteins serve as components of the mature virus particles. The mature capsids bud out of the nucleus and acquire a tegument and viral envelope with glycoprotein spikes, which are then sequentially glycosylated in transport vesicles. The release of mature virions occur via exocytosis.

4.1.3 Tropism

HHV-6 is a lymphotropic virus, but as it uses CD46 as a cellular receptor (Santoro et al., 1999), it may also infect other cells, such as monocytes, glial cells, endothelial and epithelial cells (Dagna et al., 2006). Both HHV-6A and HHV-6B replicate most efficiently in vitro in $CD4^{+}$T lymphocytes (Takahashi et al., 1989). Besides T lymphocytes, fibroblasts (Luka et al 1990), natural killer cells (Lusso et al., 1993), epithelial cells (Chen et al., 1994), endothelial cells (Caruso et al., 2002), fetal astrocytes (He et al., 1996), oligodendrocytes and microglia (Albright et al., 1998) have been successfully infected with HHV-6 in vitro. In vivo, HHV-6 infects tissues broadly. The host tissues includes brain (Chan et al., 2001, Luppi et al., 1994), liver (Härmä et al., 2003, Ishikawa et al., 2002, Ozaki et al., 2001), kidney (Gupta et al., 2003), lung (Hammerling et al., 1996, Yamamoto et al., 2005), tonsillar tissue (Roush et al., 2001), salivary glands (Fox et al., 1990) and vascular endothelium (Caruso et al., 2002).

4.1.4 Latency

HHV-6 is capable of persisting in the host after primary infection. This persistence involves both a true latent state without production of infectious virus and a low-level chronic replication. Candidate sites for latency are monocytes (Kondo et al., 1991) and early bone marrow progenitor cells (Luppi et al., 1999), whereas salivary glands and brain tissue are suspected of harboring persistent HHV-6 infection (Chan et al., 2001, Donati et al.,2003, Fox et al., 1990). HHV-6 DNA persistence has been demontrated in most immunocompetent children, wheras reactivation is found only in a few (Caserta et al., 2004). In immunocompromised hosts, it is widely recognized that HHV-6 may

reactivate and cause disease (Zerr et al., 2006). HHV-6 can reactivate from latency by superinfection with HHV-7, possible through transacting mechanisms (Katsafanas et al., 1996). There is a recognised phenomenon of HHV-6 latency characterized by integration of HHV-6 sequences into the host cell DNA, both in vivo (Daibata et al.,1999, Luppi et al., 1993, Torelli et al., 1995, Ward et al., 2006), and in vitro (Daibata et al., 1998). Moreover, congenital HHV-6 infection can be inherited from the chromosomes of one or both parents (Daibata et al., 1999). Transmission of integrated HHV-6 through stem cell transplantation has also been recently demontrated (Clark et al., 2006). Such integration is characterised by a high viral load in the blood.

4.1.5 Modulation of the host´s immune response by HHV-6

HHV-6 has potential immunomodulating properties, as the virus can induce pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-10 and IL-12 in monocytes/macrophages and downregulate CD3 in infected T-cells (Flamand et al., 1991, Krueger and Ablashi, 2003, Lusso et al., 1991). In addition, infection of PBMC has been reported to suppress T cell functions, including reduced IL-2 synthesis and cell proliferation (Flamand et al., 1995). The ability of HHV-6 to alter the expression of these key immune activation molecules lead to a changed pathogenesis associated with infection.

4.1.6 Epidemiology and transmission

HHV-6 is a ubiquitous virus, which is widespread throughout the world. Seroconversion mostly occurs by the age of 2 years, and seroprevalence in the adult population is high (> 95%) (Hall, 1997, Yoshikawa et al., 1989). Maternal antibodies are present in the neonate and decline to an undetectable level by six months of age. After this infants appear to be susceptible to primary infection. Infection can occur in the perinatal period and the virus has been found in cervical swabs from pregnant women, indicating potential vertical transmission of the virus (Okuno, et al., 1995). Based on the investigation of different anatomical sites, HHV-6 is prevalent in salivary gland tissue and in saliva (Chen and Hudnall, 2006, Fujiwara et al., 2000), Thus, the most probable route for horizontal transmission is through saliva. A serological test discriminating between the two HHV-6

variants is not available. HHV-6 is generally acquired between 6 and 15 months of age, and the incubation period is 1 to 2 weeks (Enders et al., 1990, Okuno et al., 1989). Primary infection with HHV-6 in early childhood is associated with febrile illness, including exanthem subitum (roseola infantum) (Yamanishi et al., 1988). The characteristic features of ES (*Exanthema subitum*) are typically high fever (≥ 40°C) for a few days and as the fever subsides, skin rash occurs. In cases of primary HHV-6 with variable clinical presentation such as no rash, the abrupt onset of high fever remains characteristic. The course of ES is usually benign, but some complications may occur. The most common complications are central nervous system manifestations, such as febrile seizures and encephalitis (Asano et al., 1994, Hall et al., 1994, Ishiguro et al., 1990, Suga et al., 1993). Mild liver dysfunction is also occasionally associated with primary HHV-6 infection, but cases of fulminant hepatitis have also been reported (Asano et al., 1990, Ishikawa et al., 2002, Mendel et al., 1995, Ohashi et al., 2004). HHV-6 infection with ES is almost exclusively caused by HHV-6B (Braun et al., 1997, Dewhurst et al., 1993, Hall et al., 1998). Controversely, HHV-6A is rarely identified in patients (Braun et al., 1997, Dewhurst et al., 1993). HHV-6A seroconversion is thought to occur after acquisition of the variant-B (De Bolle, et al., 2005). Some studies support the hypothesis that HHV-6A exhibits a greater neurotropism than HHV-6B (Borghi et al., 2005, Hall et al., 1998, Portolani et al., 2001). HHV-6A has also been detected in fetuses with hydrops (Ashshi et al., 2000) and in critically ill immunocompetent individuals (Razonable et al., 2002a).

4.2 HHV-6 after transplantation

Infection with HHV-6 in the post-transplant period is common and considering the high sero-prevalence in the population, it is likely to result from reactivation of the recipient`s virus or reinfection from the donor. Both HHV-6 variants A and B have been detected after transplantation, although the latter is commonly detected and HHV-6A is rare (Frenkel et al., 1994, Lautenschlager et al., 2000, Ljungman 2002). HHV-6 reactivates in 40-50% of patients undergoing haematopoietic stem cell transplantation (SCT) and in a

similar proportion of solid organ transplantation (SOT) recipients (Humar et al., 2002a, Ljungman et al., 2000, Yoshikawa et al., 1992, 2000). Following stem cell transplantation, HHV-6 reactivation is associated with a range of disease manifestations including graft versus host- disease, encephalitis, pneumonitis, and bone marrow suppression (Hentrich et al., 2005, Ljungman et al., 2000, Ogata et al., 2006, Savolainen et al., 2005, Volin et al. 2004, Zerr et al., 2006).

After organ transplantation HHV-6 infection is usually asymptomatic but has been associated with encephalitis, other infections including CMV, organ rejection and graft dysfunction (Herbein et al., 1996, Hoshino et al., 1995, Humar et al., 2002a, Lautenschlager et al., 1998, Okuno et al., 1990, Rogers et al., 2000, Singh et al., 1997). After liver transplantation, HHV-6 is common, in adult patients usually as an asymptomatic reactivation detected in 22-32 % of peripheral blood specimens of recipients (Griffiths et al., 1999, Humar et al., 2002a, Lautenschlager et al., 2000, Razonable and Paya, 2002, Schmidt et al., 1996). Although the rate of viral reactivation after liver transplantation appears to be high, the clinical relevance of these reactivations is still not completely clear. However, HHV-6 may cause fever and/or rash and other clinical symptoms, such as pneumonitis, neurological disorders, graft dysfunction and hepatitis (Herbein et al., 1996, Lautenschlager et al., 1998, Singh et al., 1995, Singh et al., 1997). HHV-6 reactivations are often seen in association with CMV-infection and allograft rejection (Griffiths et al., 1999, Humar et al., 2000, Lautenschlager et al., 1998, Lautenschlager et al., 2002b). HHV-6 causes lymphocyte infiltration and increases the expression of adhesion molecules ICAM-1 and VCAM-1 in the liver transplant, which may then lead to a local inflammation and graft damage and may even trigger allograft rejection (Lautenschlager et al,. 1998, 2002a). HHV-6 is known to have a number of cellular effects that can modulate the immunity of the host (Flamand et al., 1995). HHV-6 infection after liver transplantation has been associated with other opportunistic infections, particularly of fungal origin, but also viral infections (Dockrell et al., 1999, Rogers et al., 2000), such as CMV and HCV (Humar 2002b, Razonable et al., 2002c, Singh et al., 2002). Infections with HHV-6 typically occur in the early post-transplant period, usually within the first four weeks post transplantation (Singh and Carrigan, 1996).

4.3 Laboratory diagnostics of HHV-6

Diagnosis of primary HHV-6 infections includes standard IgG and IgM serology. Detection of viral nucleic acids may indicate active or latent infection, depending on the clinical setting and the specimen tested. Detection of HHV-6 DNA from plasma or serum is now possible and quantitative PCR methods have recently improved in their interpretability. In patients with neurological complications, viral DNA can be detected by PCR from the cerebrospinal fluid. It is also possible to culture the virus in lymphoid cell lines, but the technique is laborious and not in routine use. The accurate and rapid diagnosis of HHV-6 is essential with transplant patients. Serology is of very limited diagnostic value due to the high sero-prevalence in transplant recipients. None of the serological tests can distinguish between the antibody response to HHV-6A and B (Ward et al., 2005). Unlike antibody tests, several virus detection methods have the ability to discriminate between HHV-6A and B. Those tests include an HHV-6 antigenemia test which detects the viral antigens in peripheral blood mononuclear cells (PBMC) but this method is qualitative rather than quantitative (Lautenschlager et al., 2000). However, the HHV-6 antigenemia test is even suggested to be one of the most important methods for the diagnosis of HHV-6 in the future (Tomoiu and Flamand, 2006).

The most sensitive techniques for the detection of HHV-6 in the early period after tranplantation are those employing PCR-based techniques. However, qualitative PCR tests often fail to distinguish between latent and active HHV-6 infection, and therefore they have limited value in the diagnosis of active or productive HHV-6 infection. Some quantitative PCR-tests are available for the diagnosis of HHV-6 (Aritaki et al., 2001, Gautheret-Dejean et al., 2002, Locatelli et al., 2000, Pradeau et al., 2006) and recently also some quantitative variant-specific tests for both variants A and B have been published (Boutolleau et al., 2006, Razonable et al., 2002a, Safronetz et al., 2003). These quantitative PCR assays may offer the advantage of correlating HHV-6 viral load, similar to what has been shown in CMV infection, with clinical disease and its response to antiviral therapy. These tests have not been standardized and several specimens types, such as whole blood, PBMC and plasma, have been used. In biopsy material obtained from allografts, immunohistochemistry using monoclonal antibodies against viral

proteins or DNA in situ hybridization can be used to detect the virus (Amo et al., 2003, Härmä et al., 2006, Lautenschlager et al., 1998, Okuno et al., 1990).

4.4 Antiviral therapy for HHV-6

HHV-6 infections in immunocompetent individuals are self-limiting and do not require treatment, whereas in immunocompromised patients reactivation of latent virus may cause serious complications. There are as yet no drugs that are specifically recommended for the treatment of HHV-6 infection following transplantation. Several agents, however, have been shown in vitro to have activity against the virus, including ganciclovir, cidofovir and foscarnet (De Clercq et al., 2001). Clinical experience of antivirals in the treatment of HHV-6 infection in transplant patients is very limited. Some successful use of ganciclovir and foscarnet for symptomatic HHV-6 infection has been reported in bone marrow transplant recipients (Bethge et al, 1999, Zerr et al., 2002). When the response to antiviral therapy against CMV was investigated in liver transplant patients, reduced viral loads of HHV-6 were also recorded after ganciclovir therapy (Mendez et al 2001). On the other hand, the effect could be indirect and caused by the ganciclovir treatment of CMV infection (Humar, 2006, Mendez et al. 2001). Whether the decreased HHV-6 viral load is a result of a direct effect of ganciclovir by inhibiting HHV-6 replication or an indirect effect by first causing a decrease in CMV, which in turn will not enhance the level of HHV-6 replication, remains unclear. No controlled studies have been performed for the prevention or treatment of HHV-6 in transplant patients.

5. HUMAN HERPESVIRUS 7 (HHV-7)

Human herpesvirus-7 (HHV-7) was first isolated from $CD4^+$ T cells obtained from a healthy adult in 1990 (Frenkel et al., 1990) and is a member of the betaherpesvirus family, closely related to both HHV-6 and CMV (Berneman et al., 1992). HHV-7 is genetically most closely related to the two HHV-6 variants, with nucleic acid sequence

identity ranging from 20.7% to 75.7% in various genes (Dominquez et al., 1996, Nicholas, 1996, Megaw et al., 1998). The virus primarily infects T-cells and it uses CD4 as a cellular receptor (Lusso et al., 1994).

HHV-7 is highly prevalent worldwide, with a sero-prevalence of approximately 90%, usually acquired in early childhood and it has been associated with some cases of *exanthema subitum* (Black and Pellett,1999, Caserta et al., 1998, Tanaka et al., 1994, Torigoe et al., 1995), pityariasis rosea (Drago et al., 1997), hepatitis (Hashida et al., 1995) and neurological manifestations (Caserta et al., 1998, Torigoe et al., 1995, 1996).

Approximately 70% of children become infected during the first 5 years of life. HHV-7 is probably predominantly orally transmitted as the infectious virus is continuously shed in the saliva of healthy adults (Black et al., 1993, Fujiwara et al., 2000, Hidaka et al., 1993, Wyatt and Frenkel, 1992). Thus, salivary glands are likely sites of persistent viral infection (Sada et al., 1996, Yadav et al., 1997). After primary infection, HHV-7 establishes latency in the host and may reactivate under immunosuppression.

The role of HHV-7 in transplant patients is not clearly defined. Studies in kidney and liver transplant recipients have inferred that the interaction of HHV-6 and/or HHV-7 with CMV and the immune modulating properties are the most important factors in the illness-causing potential (Humar et al., 2000, Kidd et al., 2000, Mendez et al., 2001, Osman et al., 1996). Similar observations were also reported after bone marrow transplantations (Chan et al., 1997, Wang et al., 1996). Methods for detection of the virus include serology, detection of viral antigens and viral DNA (Black and Pellett, 1999). There are no clinical trials regarding the use of antiviral drugs for the treatment of HHV-7 infection (De Clercq et al., 2001).

6. EPSTEIN-BARR VIRUS (EBV)

6.1. EBV Virus

6.1.1 EBV genome

EBV is one of the eight known human herpesviruses. Its genome is a linear, double-stranded DNA, about 170kb in length (Farrell, 2005). Latently infected cells contain the genome as a circular plasmid in the nucleus. The terminal repeat (TR) sequences are present at both ends of the linear form of the genome and these repeats mediate the circularisation in the infected cell (Fig.2) An unusually large tandemly repeated DNA sequence in the genome of EBV is known as the major internal repeat (IR1). The IR1 site divides the EBV genome into long and short unique sequences (U_L and U_S). These sequences are filled with closely packed genes. EBV belongs to the gamma-herpesvirus family, together with human herpesvirus-8.

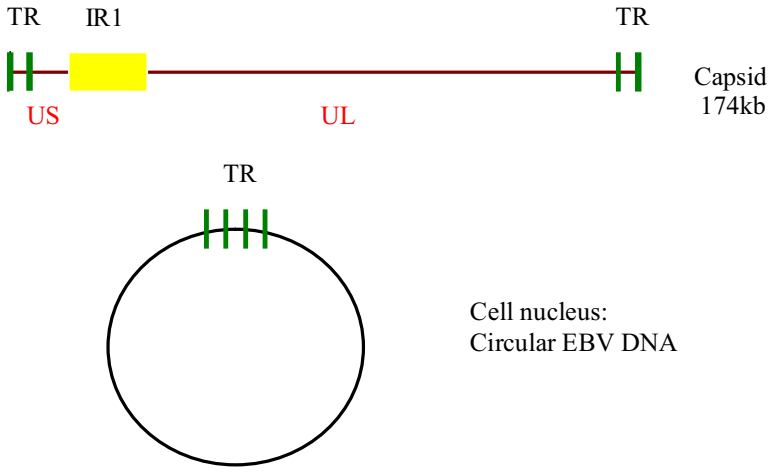


Figure 2 Diagram of the linear and circular forms of the EBV genome. TR, terminal repeat, UL, Unique long, US, Unique short, IR1, internal repeat 1. Modified from Farrell, 2005.

6.1.2 Epidemiology and transmission

EBV is a ubiquitous, worldwide pathogen, infecting more than 90% of the world`s population (Preiksaitis and Cockfield, 2003). Worldwide the seroprevalence varies depending on the population studied, from 20% to 80% by age 2 or 3 (Junker, 2005). EBV seroprevalence in Finland is over 90% among the adult population and the infection usually occurs already early in childhood, with 60% of children being seropositive by the age of six. Infection in normal individuals is often asymptomatic, though it may result in a self-limiting infectious mononucleosis in adolescence. The infectious mononucleosis syndrome is characterized by fever, lymphadenopathy, pharyngitis and an increased number of atypical lymphocytes in the blood (Cohen, 2005). In primary infections, especially in pediatric transplantations, viral transmission occurs via donor organs and blood products (Preiksaitis and Cocfield, 2003). EBV is well known to be linked to several specific human cancers such as Burkitt`s lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and some types of gastric cancers (Junker, 2005).

6.1.3 The life-cycle for EBV

EBV is spread through salivary contact and the virus enters through the epithelium that lines the nasopharynx. Infection of B-cells is initiated by the binding of the major EBV outer envelope glycoprotein gp 350/220 with the cellular complement receptor type 2 (CR2), also known as CD21 (Fig.1).The major-histocompatibility-complex (MHC) class II molecule is a cofactor for the infection of B-cells (Hutt-Fletcher, 2005). Infection results in cellular activation and immortalization. The DNA genome of EBV encodes about 100 viral proteins and during viral replication, all these proteins are expressed (Cohen, 2000). After primary infection, the EBV genome becomes circular, forming an episome in B cells, and remains latent in these cells. During primary infection, as in infectious mononucleosis, the viral antigens expressed by peripheral blood B cells are characterized by the limited expression of a subset of viral gene products, including six nuclear antigens (EBNA-1, -2, -3A,-3B, -3C, and –LP) and three integral membrane proteins (LMP-1, -2A and -2B)(Preiksaitis and Cockfield, 2003). Two small EBV-encoded RNAs (EBER-1 and EBER-2) are also found in very high copy numbers in the

nuclei of latently infected cells. In most asymptomatic carriers of EBV, the virus is occasionally replicated and infectious virions are then found in oral secretions (Tsurumi et al., 2005). The source of this virus is believed to be B cells that have become activated and circulated to the mucosal epithelium. When a reactivation of EBV infection from latently infected cells occurs, it begins with expression of immediate-early (IE) viral genes. These gene products are transcriptional transactivators that initiate the cascade of early and late gene expression leading to production of mature virion particles. Two major types of EBV strains have been recognized, EBV type-1 and type-2, and they differ biologically and in their geographic and ethnic prevalences but have no clear differences in EBV associated clinical diseases (Preiksaitis and Cockfield, 2003).

EBV is characterized by its ability to persist for the life-time of the host, although a strong humoral and cell-mediated immune response is developed in the host (Munz, 2005). Immune control of primary EBV infection is mediated by the induction of an EBV specific T-cell response, consisting of a large expansion of CD8+ cytotoxic T-cells. EBV specific CD4+ T-cells also contribute to immune control and following this cellular response, EBV is not completely eliminated but persists in memory B-cells as a lifelong asymptomatic latency. The virus has evolved strategies to elude the immune system of the host. In latent EBV infection, there are only a small number of EBV genes that are expressed at low level.

6.2 PTLD

EBV infection after transplantation is a serious problem, because of the risk of developing post-transplant lymphoproliferative disorders (PTLD) (Preiksaitis and Cockfield, 2003, Leblond and Choquet, 2004). Most PTLD cases ocurr within the first year post-transplant, when the recipient is severely immunocompromised for prevention of rejection and during periods of heavy immunosuppression (Gottschalk et al., 2005), and declines thereafter (Taylor et al., 2005).The term PTLD is used to describe a wide spectrum of lymphoproliferative disorders after solid organ and hematopoietic cell transplantation ranging from a benign self-limited form of polyclonal proliferation to

malignancies containing clonal chromosomal abnormalities (Shroff and Rees, 2004). These have been grouped into three main categories based on their clinical and histological features (Shroff and Rees, 2004). The first group consists of infectious mononucleosis and bening hyperplasia being the mildest form of syndrome. The second group is polymorphic PTLD which is an intermediate stage with polyclonal proliferation and local invasion with destruction of the nodal architecture. The third group is monomorphic PTLD where the neoplastic transformation of the tissue occurs. The EBV genome is found in most B-cell PTLD's, which occur early after solid organ transplantation, and in these early PTLD's EBV infection plays a major role in the pathogenesis of the disease (Preiksaitis and Cocfield, 2003, Shroff and Rees, 2004). However, there are late PTLD cases, which are EBV-negative, and the role of EBV in this setting is uncertain. In liver transplantations, the PTLD has been reported to occur in 0.5-2.8% of adult recipients (Niedobitek et al., 1997, Taylor et al., 2005). A higher incidence (4-15%) has been reported in paediatric liver transplant recipients, who also present more EBV primary infections. Although uncommon, the PTLD is an aggressive disease associated with high rates of mortality from 50% up to 80% (Paya et al, 1999, Taylor et al., 2005). Certain risk factors have been identified in the development of PTLD (Preiksaitis and Cockfield, 2003). EBV seronegativity of a recipient at the time of transplantation, followed by primary infection, has been recognized as a major risk factor for PTLD. Other risk factors include the type and the intensity of the immunosuppressive drugs and type of organ transplanted (Preiksaitis and Cockfield, 2003, Shroff and Rees, 2004). The development of CMV disease in liver transplant patients with a primary EBV infection has also been reported to increase the risk of PTLD (Manez et al., 1997).

EBV-induced PTLD results from an uncontrolled proliferation of B cells. This initial proliferation is polyclonal in nature, but certain clones may experience a selective growth advanced and became monoclonal ones. Malignant transformation may occur as a consequence of a cytogenetic abnormality, leading to a true malignant state (Fig.3). Naive EBV infected B cells express a repertoire of EBV proteins known as ”growth program” or latency III, which results in polyclonal B cell proliferation (Taylor et al., 2005). At least 5 of these viral proteins are essential for the transformation or immortalization process of B cells (EBNA-1, -2, 3A, -3C, and LMP-1) (Rickinson,

1998). While in healthy individual naive EBV infected B cells are killed by EBV specific cytotoxic T lymphocytes (CTL's), in an immunocompromised transplant recipient, suppression may lead to expansion of the infected cell population and increased viral shedding.

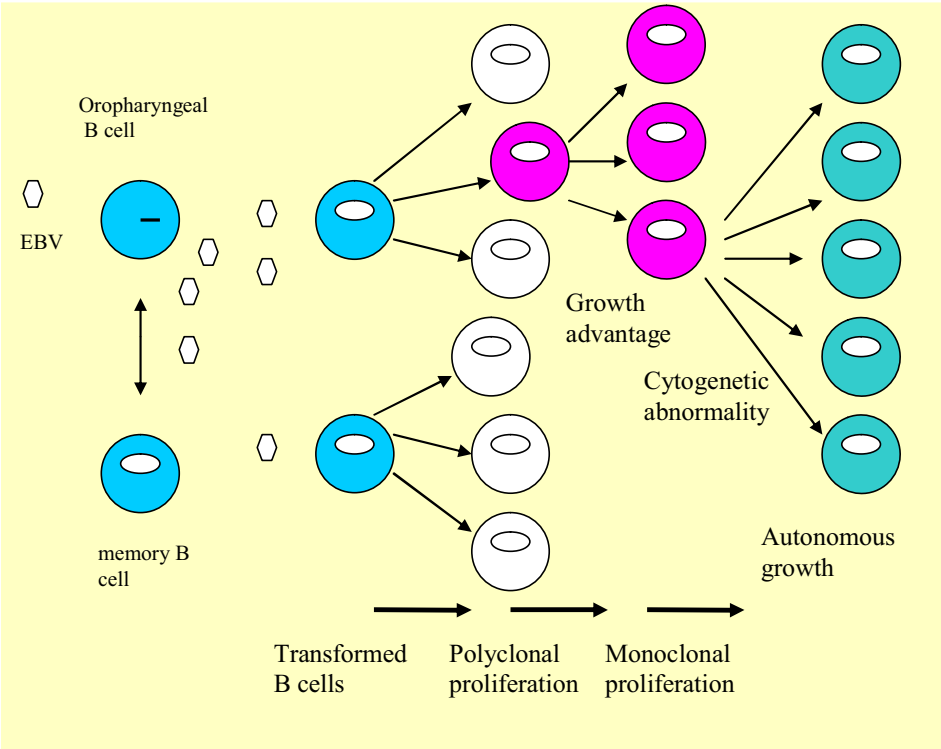


Figure 3 The pathogenesis of EBV-driven PTLD. Modified from Preiksaitis and Cockfield, 2003. In the cells, the *line* represents the lineal viral genome in lytic cycle, whereas the *halo* represents the circular, episomal EBV genome in latently infected B cell.

6.3 Diagnosis of EBV and PTLD

The determination of EBV serology in patients is usually based on enzyme linked immunosorbent assays. Detection of IgM and IgG antibodies for EBV is not useful for the diagnosis of PTLD (Granot et al., 2000, Stevens et al., 2001), as the EBV-specific

antibody response in general is weak, and the increase of EBV-specific IgM and IgG is a late event. In addition, the serological correlation to EBV activation is not ideal as most adult recipients are EBV-seropositive. Measurement of EBV viral load from peripheral blood by PCR has been evaluated as a diagnostic tool for patients with symptomatic disease. Quantification of EBV-DNA by PCR assays provide a sensitive and reliable tool for the follow-up of viral load in peripheral blood, and has been used in the clinical management of PTLD (Niesters et al., 2000, Stevens et al., 2001, Van Esser et al., 2001).

The correlation between PTLD and EBV load in whole blood, peripheral blood mononuclear cells, or by detection of tumor-derived, cell-free DNA in plasma, has been demonstrated in stem cell and bone marrow transplantation (Aalto et al., 2003, Gärtner et al., 2002, Hoshino et al., 2001, Juvonen et al., 2003), as well as in organ transplant patients (Baldanti et al., 2000, Niesters et al., 2000, Stevens et al., 2001, Tsai et al., 2002, Wagner et al., 2001). However, few studies have been published on liver transplant recipients. In one study, serial quantitative analysis of the EBV-genome was helpful in the early detection of EBV infection, and the highest EBV-DNA loads were associated with PTLD (Matsukura et al., 2002). Pediatric liver transplant recipients, mostly with primary EBV infection, developed high viral loads (Orii et al., 2000, Scheenstra et al., 2004). Higher EBV-DNA loads were seen in primary infections than in reactivations, but elevated EBV-DNA levels identified the patients at increased risk for PTLD.

The gold standard for classification of PTLD is pathological examination (Taylor et al., 2005). In addition to standard histology, the presence of EBV can be demonstrated in lymphoid tissue by immunostaining or in-situ hybridization. Further cellular infiltrates characterized by relevant phenotypic markers and clonality are determined by immunophenotyping (Taylor et al., 2005).

6.4 Treatment of PTLD

Treatment of EBV-associated PTLD consists of reduction of immunosuppression, use of anti-B-cell antibodies and chemotherapy. Although a solid tumor may be present, surgery is rarely indicated. More recently, there have been some preliminary reports of using

EBV-specific cytotoxic T-cells in stem cell transplantation (Preiksaitis and Cockfield, 2003, Taylor et al., 2005). In solid organ recipients, the reduction or withdrawal of immunosuppressive therapy can lead to reactivation of EBV-specific CTL responses in recipients and thus partial or even complete regression of PTLD (Taylor et al., 2005). Antiviral drugs, such as acyclovir and ganciclovir, inhibit only the lytic EBV infection to some degree, with no effect on the latently infected or proliferating B cells (Green, 2001). When reduction of immunosuppression fails, B cell monoclonal antibody therapy represents a good second-line therapeutic option because of its low toxicity. Anti-CD20 (Rituximab) antibody is directed against the CD20 expressed on mature and immature B lymphocytes, and it results in profound and long-lasting depletion of B cells. Chemotherapy is commonly used in combination with reduced immunosuppression in the treatment of PTLD.

AIMS OF THE STUDY

The aim of this study was to investigate CMV-, EBV- and HHV-6-DNAemia after liver transplantation from the peripheral blood specimens by regular monitoring of these patients.

The aims of the present study were in detail:

- To investigate the presence of CMV DNA in peripheral blood leukocytes after liver transplantation (I).
- To study post-transplant HHV-6-DNAemia in relation to CMV-DNAemia in liver transplant recipients (II).
- To investigate the occurrence of EBV-DNAemia, its duration and magnitude, and correlate these results with the appearance of EBV-driven PTLD (III).
- To detect HHV-6 DNA expression in PBMCs after liver transplantation and study the response to antiviral treatment by monitoring these patients (IV).

MATERIALS AND METHODS

1. Patients (I-IV)

Altogether 256 adult liver transplantations were performed between the years 1999-2005 at the Department of Surgery, Transplantation and Liver Surgery Clinic, in Helsinki University Central Hospital. The patients included in this study have been characterized in detail in the original publications I-IV and are outlined here only briefly. As basic immunosuppression, the patients received the combinations of steroids, azathioprine, and cyclosporine or tacrolimus. Rejections were treated with high doses of steroids. No routine antiviral prophylaxis was given, besides intravenous ganciclovir prophylaxis during rejection therapy. Symptomatic CMV infections were treated with intravenous ganciclovir for at least two weeks.

1.1. Blood specimens (I-IV)

The clinical material comprised of EDTA-blood samples obtained weekly during the patients´ hospitalization and thereafter, according to our clinical protocol, at 1, 2, 3, 6, 9 and 12 months after transplantation and in case of clinical symptoms. The blood sampling for the CMV-DNA in situ hybridization, HHV-6-DNA in situ hybridization, HHV-6 DNA-PCR and EBV DNA-PCR occurred in parallel with sampling for the CMV-pp65 antigen test, CMV DNA-PCR and HHV-6 and HHV-7 antigen detection, which were performed immediately. Plasma and blood samples and cytocentrifuged cellular specimens were stored at -70°C for later retrospective analysis of EBV DNA-PCR, HHV-6 DNA-PCR and CMV-and HHV-6- in situ hybridizations.

2. Detection of CMV

2.1. CMV antigenemia test (I, II, III)

CMV antigenemia was monitored from the peripheral blood leukocytes using the standard CMV pp65 antigenemia test (The et al., 1995). The leukocytes were isolated from blood samples and cytocentrifuged onto microscope slides. The slides were dried at room temperature and fixed in cold acetone. The CMV positive cells were demonstrated by immunoperoxidase staining using a monoclonal antibody against CMV pp65 antigen (Biotest AG, Dreieich, Germany). The results were quantified by counting the number of pp65 postive cells per 50 000 leukocytes on the slide according to the descriptions of the original methods (The et al., 1995, Van der Berg et al., 1989).

2.2. CMV in situ hybridization assay (I)

The hybridization procedure was a modification (Lautenschlager et al., 1997) of the previously described protocol (Brigati et al., 1983). CMV in situ hybridization was performed from peripheral blood leukocytes using a biotinylated DNA-probe (Enzo, Farmingdale, NY). According to the manufacturer the probe was composed of two BamHI fragments derived from the Towne strain of CMV, each cloned into a plasmid vector. The probe was prepared from a mixture of two clones of CMV sequences in the BamHI site of pBR22. The insert sizes were 13.3kb and 16.6kb. The fragments include a total of 30-31kb of DNA, approximately 20% of the cytomegalovirus genome. In parallel, a negative reagent control of hybridization mixture without the probe was also performed for each specimen. After hybridization, the slides were washed under stringent conditions. A rabbit anti-biotin antibody (Enzo, Farmingdale, NY) followed by a second biotinylated anti-rabbit IgG (Vector laboratories, Burlingame, CA), and streptavidin-alkaline phosphatase conjugate (Gibco BRL, Gaithersburg, MD) was used for detection. NBT-BCIP (Boehring Mannheim, Mannheim, Germany) was used as a substrate and Hemalum was used as a counterstain. The positive reaction of the in situ hybridization

was seen as a spotted, dark, nuclear DNA staining. The number of DNA-positive cells was counted per 50 000 leukocytes.

2.3. CMV quantitative PCR (II, III)

Quantitative DNA-PCR, Cobas Amplicor CMV Monitor, Roche, or real-time TaqMan PCR was used for quantitation of CMV-DNA in plasma specimens as described previosly (Piiparinen et al., 2002, Piiparinen et al., 2004). The DNA isolation from 200µl of plasma was performed either by lysis of virus particles followed by alcohol precipitation or recently by using the automated MagNa Pure LC instrument (Roche Diagnostics). The linear range of the Cobas Amplicor CMV Monitor-assay was 400-100,000 copies/ml (cps/ml) according to the manufacturer's recommendations and in real-time TaqMan based PCR it was 250- 25,000 000 genome equivalents (ge)/ml.

3. Detection of HHV-6/ 7 (II, III, IV)

3.1. HHV-6 and -7 antigenemia tests

For the HHV-6 and -7 antigenemia tests, PBMC were isolated by Ficoll-Paque density gradient centrifugation and cytocentrifuged onto microscope slides. HHV-6 antigenemia test was performed on PBMCs using immunoperoxidase staining and monoclonal antibodies (Chemicon, Temecula, CA, USA) against an early HHV-6 antigen (variants A and B), and HHV-6B virion protein, as described by Lautenschlager et al. (2000). HHV-7 specific antigens were detected in the cytocentrifuged PBMC preparations by immunoperoxidase staining with two monoclonal antibodies (Biodesign, Saco, ME, USA) detecting the early and late (gp110 and gp160) HHV-7 antigens, respectively (Lautenschlager et al., 2002b).

3.2. HHV-6 in situ hybridization assay (IV)

HHV-6 DNA was demonstrated from the same PBMC specimens by in situ hybridization using a biotinylated oligoprobe-mixture detecting both HHV-6 variant A (5`-AAAACATTGAAGAAGTTT-3`) and variant B (5`-AAGACATTGAAGAAGCTT-3`) (Qiagen, Cologne, Germany). The HHV-6 hybridization procedure was a modification of previously described protocol (Yadav et al., 1996). After hybridization, HHV-6 DNA was demonstrated using rabbit-anti-biotin antibody, followed by a biotinylated anti-rabbit-IgG antibody and streptavidin-alkaline-phosphatase conjugate. NBT-BCIP was used as a substrate and Hemalum as a counterstain. The HHV-6 in situ hybridization findings were quantified as DNA positive cells/ 10 000 PBMC.

3.3. HHV-6 real-time PCR (II)

TaqMan based quantitative PCR was used for quantitation of HHV-6 DNA in blood specimens. The DNA isolation from 500µl of blood was performed by lysis of virus particles followed by alcohol precipitation. The primers 1 (5´-CGAAGCGGTAA AACTGCGT-3´) and 2 (5´- CGATCATTCTCAACCTAGCGC-3´) amplified a 68bp length product in a U67 gene of HHV-6. A specific probe for HHV-6 (5´TCAGTGTGTAGTT CGGCAGCCCCG-3´) was used. The primers and the probe were synthesized by Sigma Genosys. The PCR amplification was performed in a 50-µl volume containing 1 x TaqMan Universal PCR Master Mix (PE Applied Biosystems), 0.30µM of each primer, 0.25µM of the fluorogenic probe and 10µl of the isolated DNA. All the samples were run in duplicate. The amplification was carried out in ABI PRISM 7700 sequence detector (Applied Biosystems). The standard curve was created using the ABI PRISM 7700 Sequence Detection System software by plotting the CT values against the known HHV-6 DNA concentrations. The first step of the amplification program was 2 min at 50°C. In this step, uracil- N-glycosylase, which is included in theTaqMan Universal Mix, destroys the possible contaminating preamplified products containing deoxyuridine. In the next step, an incubation of 10 min at 95°C allowed the activation of AmpliTaq Gold enzyme and the denaturation of nucleic acids. Forty-five cycles of

denaturation at 95°C for 15 seconds and annealing-extension at 60°C for 1min were then carried out, allowing the amplification of HHV-6 DNA.

4. Detection of EBV by real-time PCR (III)

EBV DNA was isolated from plasma by using the MagNa Pure LC Instrument (Roche). MagNa Pure LC Total nucleic acid isolation kit (Roche) was used for extraction of total nucleic acids from plasma samples. The nucleic acids were eluted in 50µl of low salt elution buffer and 12.5 µl of the eluted nucleic acid was used for real time PCR, modified from the method described earlier (Aalto et al., 2003). The primers amplified a conserved sequence of the viral DNA polymerase (BALF5) gene, which has been described previously (Kimura et al., 1999). The amplification was carried out in the ABI PRISM 7900HT sequence detector (Applied Biosystems). The standard curve was created with the ABI PRISM 7900HT Sequence Detection System software by plotting the CT (cycle threshold) values against the known EBV DNA concentrations (Commercial EBV-DNA, strain B95-8, Advance Biotechnologies Incorporated, Columbia, MD, USA). The detection limit of the EBV-DNA-PCR was 500 copies/ml plasma (Aalto et al., 2003).

5. Statistical methods (I-IV)

Data are expressed either as means$\pm$ SD (I,II,IV) or as medians (III). For statistical analysis, non-parametric Wilcoxon Signed-Rank Test was used and P-values < 0.05 were considered to be significant (III).

RESULTS

1. CMV DNAemia after liver transplantation (I)

The presence of CMV-DNA in peripheral blood leukocytes was frequently monitored by in situ hybridization and compared with CMV pp65-antigenemia in 20 liver transplant recipients during the first six months after transplantation. Eleven patients out of 14 with CMV infection had a regular follow-up in our hospital and received antiviral ganciclovir treatment for a minimum of two weeks. The dose was 10 mg/kg b.w./day and this was adjusted in case of kidney dysfunction. The total number of blood specimens in this study was 202.

CMV pp65-antigenemia was detected mean on day 33 after transplantation. CMV DNA was detected a mean 15 days earlier than CMV pp65-antigenemia and the peak CMV-antigenemia levels correlated well with the peak CMV-DNA levels. All treated patients responded to ganciclovir and the CMV-antigenemia subsided. However, there were eight patients, who showed continuous low level CMV DNA positivity in their peripheral blood leukocytes for up to six months. In six patients, a recurrent CMV infection with pp65 antigenemia occurred during the follow-up but the CMV disappeared in response to antiviral treatment in all six cases. In these patients, the CMV DNAemia persisted right up until the next recurrence. The follow-up of CMV pp65 angenemia and DNAemia of one patient is demonstrated (Fig.4).

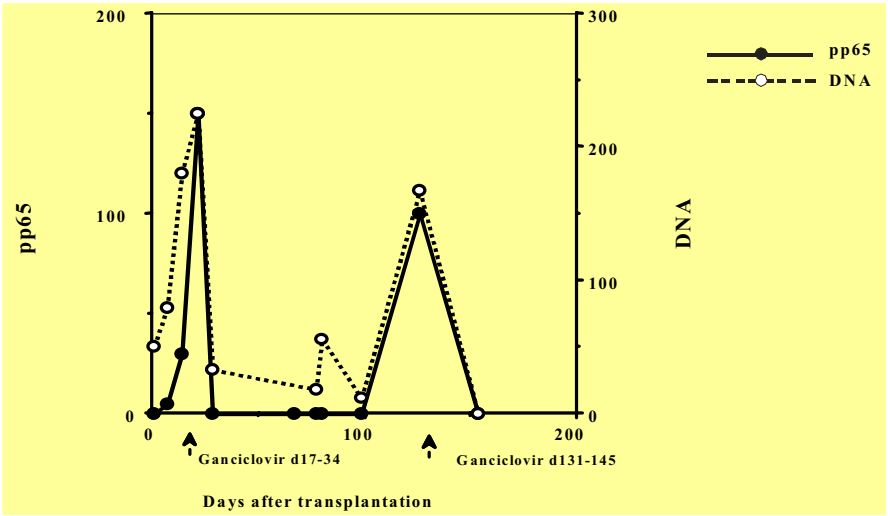


Figure 4 The follow-up of a patient with two episodes of CMV infections treated with ganciclovir.

2. HHV-6 DNAemia after liver transplantation (II, IV)

HHV-6 DNAemia was detected by a quantitative PCR test and by DNA in situ hybridization assay. In the HHV-6 DNA-PCR study (II), the purpose was to investigate post-transplant HHV-6 DNAemia in relation to CMV-DNAemia in liver transplant patients. HHV-6 DNA in situ hybridization assay (IV) was used to study the appearance of HHV-6 DNA expression in PBMC, to quantitate the cellular findings and to assess the potential usefulness of this method as an alternative tool in the monitoring of liver transplant patients. At the same time the response to ganciclovir treatment was also investigated.

2.1 HHV-6 DNA-PCR (II)

Thirty-one adult liver allograft recipients were regularly monitored for CMV and HHV-6 during the first 3 months after transplantation. Altogether 253 blood specimens were analyzed. Thirteen patients (40%) developed symptomatic CMV infection, mean on day 33 after Tx (range 5-62), and were treated with intravenous ganciclovir. The peak viral loads of these symptomatic CMV infections were high (CMV DNA 34210±37557 copies/ml), whereas six additional asymptomatic patients demonstrated significantly lower CMV DNA-levels (1020±1008 copies/ml, $p < 0.05$) and were not treated. Concurrently with CMV, HHV-6 DNAemia / antigenemia was detected in 17 of 19 patients, with first positive finding mean on day 11 after transplantation. In most cases HHV-6 appeared before CMV (12/17). However, the peak HHV-6 viral loads were low (< 1500 copies/ml blood), even in those five patients who demonstrated HHV-6 antigen at liver biopsy. All CMV infections responded to ganciclovir treatment and the CMV DNAemia/antigenemia subsided. HHV-6 also responded to the antiviral treatment, but much more slowly.The overall CMV/HHV-6 findings of treated and not treated patients are shown (Table 1).

	HHV-6 +	**HHV-6 -**	**TOTAL**
CMV + (CMV treated)	**17 (12)**	**2 (1)**	**19 (13)**
CMV-	**6**	**6**	**12**
TOTAL	**23**	**8**	**31**

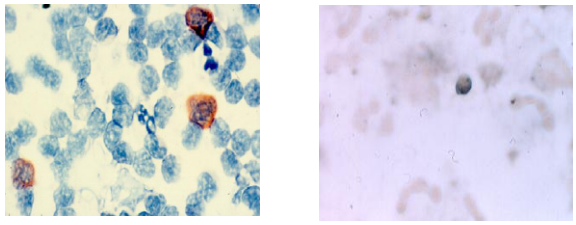
Table 1 CMV antigenemia/DNAemia related to HHV-6 antigenemia/DNAemia. The overall CMV and HHV-6 findings of treated and not treated patients.

2.2. HHV-6 DNAemia by in situ hybridization (IV)

During the first six months of monitoring after transplantation, 35/43 recipients developed HHV-6 antigenemia mean on day 12 (range 2-28 days). Concurrent HHV-6 DNA expression in PBMC was detected in 33/35 patients with a mean peak number of 661 HHV-6 DNA positive cells/10,000 PBMCs (Fig.5). Two patients with HHV-6 antigenemia were HHV-6 DNA in situ negative.

Seven patients received ganciclovir treatment because of concurrent symptomatic CMV infection with mean peak number of HHV-6 positive cells 381/ 10,000 PBMCs before and 34/ 10,000 after the treatment (p=0.03). In these patients, HHV-6 antigenemia was recorded earlier (mean on day 9) than CMV antigenemia (mean on day 19). All CMV infections were successfully treated with intravenous ganciclovir and CMV antigenemia subsided. HHV-6 antigenemia lasted in ganciclovir treated patients a mean 24 days (range 4-79) and HHV-6 DNAemia a mean 36 days (range 4-79). The follow-up of one patient with ganciclovir treatment is shown in fig.6.

In patients who did not receive antivirals due to CMV, the HHV-6 antigenemia and DNAemia episode usually lasted longer than in those with ganciclovir treatment, but also these patients also became HHV-6 DNA negative within six months after transplantation. The mean peak number of positive cells in these patients was 750/ 10,000 PBMC. HHV-6 antigenemia disappeared slowly, mean on day 51 and HHV-6 DNA mean on day 43.



a) b)

Figure 5 a) HHV-6 antigen positive peripheral blood mononuclear cells demonstrated by immunoperoxidase staining and b) a HHV-6 DNA positive cell demonstrated by *in situ* hybridization (original magnification x 1000).

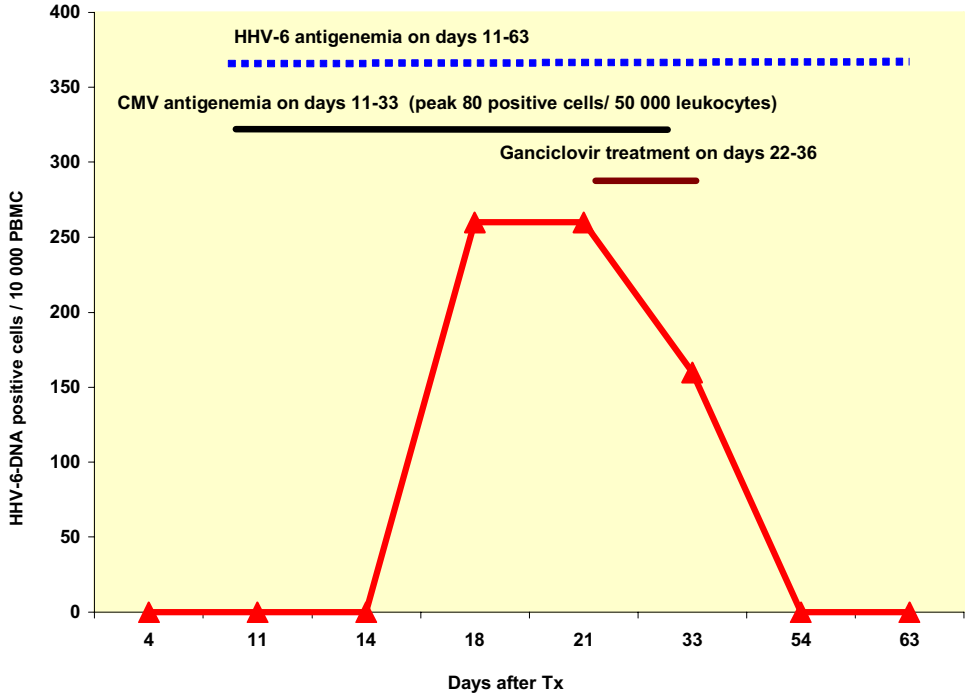


Figure 6 The follow-up of a patient with ganciclovir treatment

3. EBV DNAemia after liver transplantation (III)

The EBV-DNA levels were quantified from sequential plasma specimens, which were obtained from 105 adult liver transplant recipients during the first year after transplantation. Altogether, 14/105 patients (13%) showed EBV-DNAemia, which usually occurred within the first three months after transplantation. In 10/14 patients, EBV-DNAemia occured together with CMV; in 11/14 patients together with HHV-6; and in 4/14 patients together with all three betaherpesviruses. All CMV infections were succesfully treated with ganciclovir. HHV-6 and HHV-7 also responded to ganciclovir or the antigenemia subsided slowly without antivirals.

The peak viral EBV loads of 13 patients were relatively low (median 2100 EBV-DNAcopies/ml plasma, range 568-6600), and the EBV DNAemia usually disappeared within a few weeks after transplantation (Fig.7). During the first year, ten of these patients had one EBV DNA episode, whereas another three patients had two episodes. No clinical signs or symptoms could be attributed to these EBV DNAemias. Thus, all these low-level EBV episodes were temporal, transient and clinically harmless reactivations of the virus.

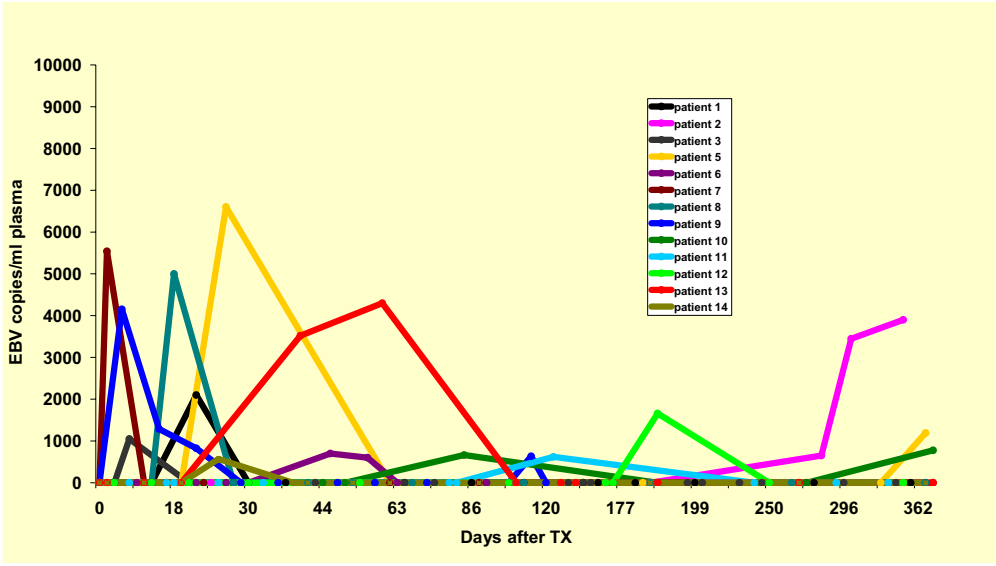


Figure 7 Viral loads in plasma of the 13 liver transplant recipients with low copy numbers of EBV-DNA.

One patient developed PTLD six months after liver transplantation. Liver transplantation was performed on a 49-year-old man with alcoholic liver cirrhosis. Six months after transplantation, the patient developed jaundice. This PTLD patient, who was EBV-seropositive before transplantation, initially showed low-level EBV DNAemia on day 22 after transplantation (562 copies/ml), remained negative thereafter until day 49, and again became positive at day 70 (755-3022 copies/ml)(Fig.8). On day 175, he developed a new EBV DNA episode, and now with high copy numbers (16,292 copies/ml), which

continued for six months (peak 86,975 copies/ml) (Fig.8). Six months after transplantation, the patient developed a neoplastic lesion of the pancreas and infiltration into the surrounding tissues. Histological biopsy showed PTLD, predominantly of B-cells with severe proliferation. The pancreatic tumor was positive for EBV at immunohistochemistry and by in situ hybridization.The immunosuppressive therapy was interrupted and the patient received anti-CD20 treatment, radiation therapy and chemotherapy. The viral loads decreased somewhat during the anti-CD20-treatment from 81,000 copies/ml to 31,300 copies/ml (Fig.9). The therapies were interrupted for serious gastrointestinal bleedings from tumor and septicemia.The patient died six months after the PTLD diagnosis.

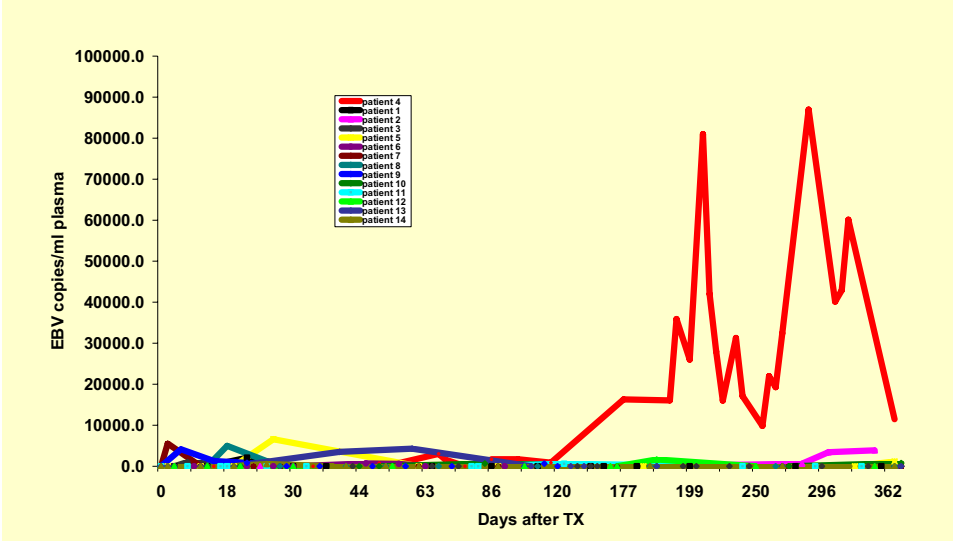


Figure 8 Viral loads of all 14 liver transplant recipients with positive EBV-PCR results. The patient with PTLD is shown red.

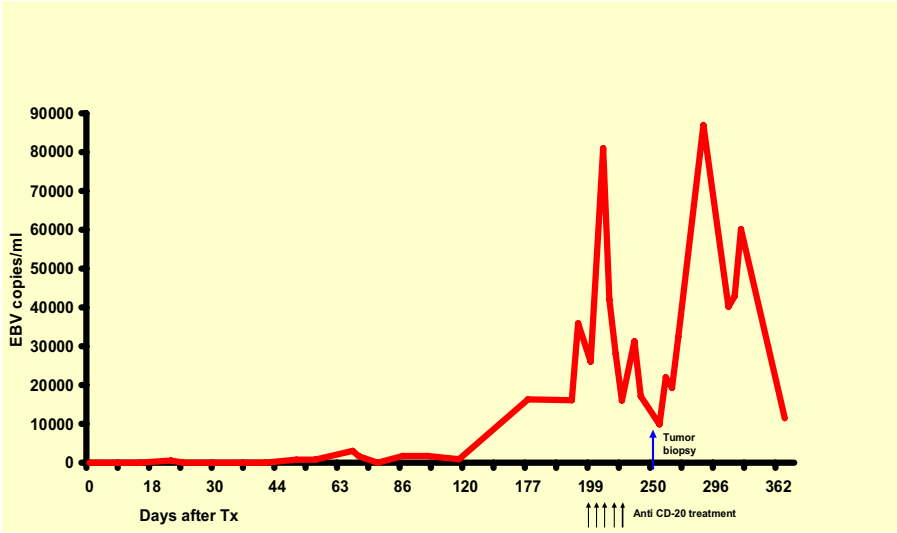


Figure 9 Viral loads of the PTLD patient. The black arrows show the anti-CD20 antibody treatment and the blue arrow show the timepoint of tumor biopsy.

DISCUSSION

Viral infections are common complications after liver transplantation and these infections are important to recognize and treat early, as they may cause severe complications. Herpesviruses remain latent in the body after primary infection and periodically reactivate under immunosuppressive conditions, such as transplantation, making them important pathogens in transplant recipients. CMV remains amongst the most significant opportunistic pathogens in liver transplant recipients causing the most severe disease. Recently, significant advances have been made in the management of CMV infection in transplant recipients, including the development of more sensitive diagnostic tests, and the prophylactic and pre-emptive antiviral strategies to prevent CMV disease. The role of HHV-6 and HHV-7 as pathogens in transplant recipients is less well understood. Besides direct clinical effects, these viruses may have indirect effects, such as interaction with CMV. However, further studies are required to establish their real impact in liver transplantation. EBV infection after transplantation is a serious problem because of the development of PTLD, which is a rare, but a serious complication.

1. CMV DNAemia after liver transplantation

CMV infections are common after liver transplantation, but usually successfully treated with antivirals. In the first study, the presence of CMV-DNA in peripheral blood leukocytes was monitored by in situ hybridization and compared with CMV pp65-antigenemia in liver transplant patients receiving ganciclovir treatment. Monitoring of these patients by frequently obtained peripheral blood specimens demonstrated that the appearance of CMV DNA precedes CMV pp65 antigenemia. This study demonstrated also that CMV DNA was not abolished by ganciclovir, although the patients were successfully treated with the antiviral drug and CMV pp65 antigenemia subsided. Thus, recurrences can be expected in liver transplant recipients during the subsequent months.

This kind of persistence of CMV DNA in peripheral blood leukocytes even up to six months after ganciclovir treatment, has not been described earlier.

CMV-pp65 antigenemia is widely used for monitoring the response to antiviral treatment of CMV disease (The et al., 1995, Boeckh and Boivin, 1998). Although most patients respond during the two weeks treatment and CMV antigenemia disappears, some patients need longer treatment when guided with the monitoring of pp65. Even if the patient becomes CMVpp65 negative during treatment, the viral DNA expression may continue and recurrences are possible. Thus, the response in pp65 antigenemia may be misleading and the monitoring of CMV DNA demonstrates better the temporal persistence of the virus.

Today, most centers have programs, not only for treatment of CMV disease, but also for pre-emptive therapy and prophylaxis of CMV. Ganciclovir and its derivate valganciclovir are effective in preventing viral replication (Singh 2005, Paya et al., 2004) but they have no effect on the viral genome or on the latent virus. In most centers the patients are also monitored by other means than CMV pp65 antigen test. The diagnosis of CMV infection and disease has evolved considerably in recent years, with demonstration of the viral DNA by quantitative PCR tests. These sensitive tests have shown a good clinical correlation in diagnosing CMV infection and in the monitoring of viral load after transplantation (Humar et al.1999, Meyer-Koenig et al., 2004, Piiparinen et al., 2001, 2003, Rollag et al., 2002, Tong et al., 2000).

2. HHV-6 DNAemia after liver transplantation

In addition to CMV, activation of other betaherpesviruses, especially HHV-6, has been reported after liver transplantation. In study II, the posttransplant HHV-6-DNAemia was investigated in relation to CMV-DNAemia in liver transplant recipients. After liver transplantation, detection of HHV-6 in the blood, by culture or PCR, has been reported in 28% to 45% of patients (Herbein et al., 1996, Schmidt et al., 1996), and the pathogenicity of the infection has been described (Singh et al., 1997). Although the rate of viral

reactivation after liver transplantation appears to be high, the clinical relevance of these reactivations is still not completely clear. In this study, concurrently with CMV, HHV-6 DNAemia/antigenemia was commonly detected in peripheral blood after liver transplantation. In time scale, HHV-6 appeared prior to CMV in most cases. HHV-6 DNAemia and antigenemia correlated in all patients. However, the HHV-6 peak viral loads were low in all cases, even in those five patients who demonstrated HHV-6 antigens at liver biopsy. This might be explained by the tropism of HHV-6 and its intracellular nature in the mononuclear cells. In contrast, CMV replicates actively in many types of cells, such as epithelial and especially endothelial cells, and large amounts of the virus are shed in the peripheral blood (Grefte et al. 1993).

CMV DNAemia/antigenemia subsided with ganciclovir treatment but HHV-6 DNAemia/ antigenemia tended to last longer. In vitro, ganciclovir, foscarnet and cidofovir all have antiviral activity against CMV and HHV-6. As HHV-6 reactivations are usually asymptomatic, it is difficult to prove but ganciclovir treatment may have had some effect against HHV-6, although not as much as against CMV. However, in vitro foscarnet has been shown to be the most effective agent against HHV-6, whereas the most effective against CMV is ganciclovir (De Clercq et al., 2001). Both ganciclovir and foscarnet have been reported to be effective in the treatment of HHV-6 meningo-encehalitis after stem cell transplantation (Ljungman, 2006).

In study IV, the detection of HHV-6 DNA in PBMC was compared with HHV-6 antigenemia in liver transplant patients and it was found that HHV-6 DNAemia correlated well with HHV-6 antigenemia in these patients. The HHV-6 in situ findings were quantified and correlated with HHV-6 antigenemia. The HHV-6 antigenemia test, which is not quantitative, has also been used as an alternative method for the diagnosis of an active HHV-6 infection in transplant recipients (Lautenschlager et al., 2000, Savolainen et al., 2005, Volin et al., 2004). Diagnostic results may be affected by the fact, that high HHV-6 DNA levels in whole blood and serum are found in individuals with viral chromosome integration (Ward et al, 2006). However, integrated viral DNA was not found in our study, because all the HHV-6 DNA positive recipients became negative within six months after liver transplantation.

The response to antiviral treatment was also investigated. Concurrent CMV infection occured in seven patients, who were then treated with ganciclovir. In these patients, the peak number of HHV-6 DNA positive cells was significantly higher before than after the treatment, demonstrating the positive response to ganciclovir. Also the HHV-6 antigenemia/DNA episode was somewhat shorter compared with the patients who did not receive antivirals. Thus, viral response to ganciclovir treatment was recorded.

Clinical experience with antivirals in the treatment of HHV-6 infections in transplant patients is limited. No controlled study has been conducted for prevention or treatment of HHV-6 in transplant patients. In one previous study the response to antiviral therapy against CMV was investigated in liver transplant patients. In that study, reduced viral loads of HHV-6 were also recorded after ganciclovir treatment (Mendez et al., 2001). However, the effect could be indirect and caused by the ganciclovir treatment of CMV infection (Humar, 2006, Mendez et al., 2001). CMV interacts with other beta-herpesviruses and may stimulate HHV-6. Thus, eradication of CMV with ganciclovir treatment may in addition abolish HHV-6. Prospective clinical trials using various antivirals for prophylaxis or treatment of betaherpesviruses would prove the possible clinical effectiveness of these drugs against HHV-6 in solid organ transplantation.

3. EBV DNAemia after liver transplantation

In study III on adult liver transplant recipients, a low-level EBV-DNAemia was common and usually occurred shortly after transplantation, together with other herpesviruses. These low level EBV DNA episodes seemed to be clinically harmless reactivations, which subsided within a few weeks. Reactivation of betaherpesviruses was frequently recorded during the early post-transplant period. These reactivations were not clearly more frequently in recipients with EBV DNAemia than in those without. Thus, reactivations of betaherpesviruses did not necessarily correlate with an increased risk of EBV-DNAemia.

Various diagnostic procedures have been developed for identification of patients at risk of PTLD. While these new quantitative PCR assays are promising, they have several limitations. For example these tests are not standardized, and various blood components have been used in different studies. In this study, one patient developed EBV-driven PTLD, with high copy numbers in plasma. It has previously been demonstrated in transplant recipients that high EBV viral loads in PBMC preceded the development of PTLD (Kimura et al., 1999), whereas lower level of EBV-DNA without signs of EBV-PTLD occurred in the same proportion of recipients as in our study. Renal transplant recipients with PTLD had a median viral load significantly higher than in other recipients with EBV (Wagner et al., 2001). The diagnostic specificity was also found to be higher with plasma than PBMC. In our study, the low level EBV DNAemia in plasma specimens of adult liver transplant recipients confirms the results obtained in previous studies (Niesters et al., 2000, Wagner et al., 2001), as did the peak viral load of the patient with EBV-PTLD. The same TaqMan based real-time EBV-PCR is highly sensitive (100%) and specific (96%) for evaluating stem cell recipients (Aalto et al., 2003, Juvonen et al., 2003). The quantitative plasma PCR-method used in this study was developed for the monitoring of stem cell transplant patients and has been successfully employed with the adult population to detect patients at the risk for PTLD (Juvonen et al. 2003).

The EBV-induced PTLD results from an uncontrolled proliferation of B cells. Most of these cases occur within the first year post-transplant, when the recipient is severely immunocompromised. However, only in a minority of transplant recipients this uncontrolled EBV-driven proliferation leads to formation of tumor and the onset of PTLD. It has been found that CMV disease increases the risk of developing PTLD when primary EBV infection occurs after liver transplantation (Manez et al., 1997). In our study only one patient developed EBV-PTLD and this patient was EBV-seropositive prior to transplantation and there were no other predictors, such as CMV, either.

The early diagnosis and treatment of PTLD are important for a favourable clinical outcome. Reduction or withdrawal of immunosuppression remains the cornerstone of PTLD therapy. On the other hand, treatment with anti-CD20 antibody is effective in many cases. Chemotherapy is currently the treatment of choice for aggressive PTLD.

The antivirals are not active against B cells which are latently infected with EBV. However, antiviral drugs may have some effect on the minority of lytically infected cells within PTLD lesions and prevent spreading of EBV to new clones of previously uninfected B-cells. Longitudinal monitoring of the EBV viral load in the peripheral blood of transplant recipients is a valuable tool for the prediction, diagnosis and therapeutic management of PTLD. Our findings indicate that frequent monitoring of EBV viral loads by quantitative PCR may be helpful in the follow-up of patients and in detection of the possible, though rare, adult recipients at risk of developing PTLD. In consideration of the cost-benefit ratio, these patients could be tested for EBV-DNA once a month during the first post-transplant year and, if there are any signs of EBV-DNAemia, then switched to more frequent monitoring. However, a cost effective analysis should be performed for definitive recommendations for such a monitoring regimen. In any case, further prospective studies are needed to standardize EBV viral load quantitation between the centers and developing appropriate positive predictive values.

4. Conclusions

The detection of CMV-DNA in peripheral blood leukocytes precedes CMVpp65 antigenemia in liver transplant patients. Continuous CMV-DNA expression of peripheral blood leukocytes demonstrates that the virus is not eliminated by ganciclovir treatment and recurrences can, in spite of successful anti-viral treatment of CMV disease and subsiding of CMVpp65 antigenemia, still be expected during several months after liver transplantation.

HHV-6 DNAemia/antigenemia was common and usually associated with CMV in liver transplant recipients. HHV-6 DNAemia correlated with HHV-6 antigenemia. CMV DNAemia/antigenemia subsided with ganciclovir treatment but HHV-6 DNAemia/ antigenemia tended to last longer.

Detection of cellular HHV-6 DNA by in situ hybridization correlated well with HHV-6 antigenemia and may be used as an alternative tool in the monitoring of liver transplant recipients. Antiviral treatment significantly decreased the number of HHV-6 DNA positive cells, thus demonstrating the response to ganciclovir treatment. Further investigation of the clinical significance of HHV-6 DNAemia, and the response to antiviral treatment of HHV-6 infection after liver transplantation, is necessary.

Clinically silent EBV reactivations with low viral loads were relatively common after liver transplantation in adults. EBV DNAemias usually occurred, in connection with reactivation of other herpesviruses within three months after transplantation. The EBV DNAemia subsided without treatment within a few weeks. However, one patient developed PTLD with high viral loads. Our findings indicate that frequent monitoring of EBV viral loads can be useful to detect liver transplant patients at risk of developing PTLD.

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