Chapter 2

The value of prospective monitoring of Epstein-Barr virus DNA in blood samples of pediatric liver transplant recipients

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Abstract

Background
Post-transplant lymphoproliferative disease (PTLD) is one of the major causes of morbidity and mortality in liver transplant patients. A primary Epstein-Barr virus (EBV) infection is a major risk factor for developing PTLD.

Aim
The aim of this study was to determine circulating Epstein Barr virus (EBV) DNA after liver transplantation (LTx) in pediatric patients in relation to primary EBV infection and development of post transplant proliferative disease (PTLD).

Patients and methods
All pediatric patients who underwent LTx in 2000 and 2001, and survived the first 6 months, were included in the study. EBV serology was performed before LTx. Every 4 weeks after LTx a competitive quantitative polymerase chain reaction (PCR) assay for EBV nuclear antigen-1 was performed. Patients were followed for development of a PTLD.

Results
Thirteen patients were included in the study. Before LTx 4 patients were EBV positive and 9 patients were EBV seronegative. In one of the 4 patients who were EBV seropositive before LTx, EBV DNA became detectable after LTx, with a peak load of 3600 copies/ml. None of these 4 patients developed a PTLD. Eight of the 9 patients who were EBV seronegative before LTx developed positive EBV DNA samples. EBV DNA was first detected at a median of 64 days after LTx (range 38-89). The median peak EBV DNA load was 20,600 copies/ml (3600-446,000). Two of these patients developed PTLD, but they could not be identified based on prior or concomitant EBV PCR results.

Conclusions
In pediatric LTx EBV DNA load is higher in patients with a primary infection than in patients who were EBV seropositive before LTx. The EBV PCR cannot be used to identify individual patients who develop PTLD. However, elevated EBV DNA load can be used to detect a group of patients at increased risk for PTLD.

Abbreviations
Epstein-Barr virus = EBV
Liver transplantation = LTx
Post-transplant proliferative disease = PTLD
Polymerase chain reaction = PCR
Cytomegalovirus = CMV
Epstein-Barr nuclear antigen = EBNA
Introduction

The Epstein-Barr virus (EBV) is related to the development of post-transplant lymphoproliferative disease (PTLD) in immunosuppressed patients.\textsuperscript{1,2} The probable pathophysiological mechanism underlying PTLD is imbalance between EBV-induced B lymphocyte proliferation and T cell surveillance.\textsuperscript{3} PTLD is characterized by uncontrolled B lymphocyte proliferation after LTx.\textsuperscript{4} PTLD is one of the major causes of mortality and morbidity after the direct postoperative period among children undergoing solid organ transplantation. The incidence of PTLD after liver transplantation (LTx) is much higher in children than in adults and ranges from 3 to 20%.\textsuperscript{5} The high incidence of PTLD in children may be a result of the predominant occurrence of primary EBV infections after LTx in this age group, which is one of the important risk factors for developing PTLD. Most children do not undergo an EBV infection before LTx, and frequently receive a (partial) graft from an adult donor in which EBV resides. As a consequence, most pediatric LTx patients undergo a primary EBV infection in the first months after LTx, in the period with the highest level of immunosuppression.

Presentation of PTLD varies from a relative benign proliferation of B lymphocytes to a malignant lymphoma. The cornerstone of treatment of PTLD involves reduction of immunosuppression, for a more effective T cell response against the EBV-infected, proliferating B cells. Treatment is not without risk; however, because reduction of immunosuppressive treatment increases the risk of rejection of the transplanted organ. Pre-emptive strategies such as immunomodulation in risk groups to prevent PTLD have been described.\textsuperscript{5,6} To enable a pre-emptive strategy early recognition of patients at risk is important. A high EBV DNA load in the peripheral blood has been associated with an increased risk on PTLD.\textsuperscript{7-10} Recently, tools have become available to quantitate EBV DNA load in peripheral blood by using a quantitative polymerase chain reaction (PCR) for EBV. It has remained unclear, however, if serial determination of EBV DNA load in peripheral blood of pediatric liver transplant patients allows identifying patients at high risk for the development of EBV-associated disease. To estimate the diagnostic potency of the quantitative EBV DNA load, longitudinal studies are warranted.

In the present prospective study we determined the relationship between the circulating EBV DNA load in the first 6 months after LTx and primary EBV infection and development of PTLD in pediatric patients. We investigated the relationship between EBV DNA load and clinical or biochemical parameters. We analyzed whether patients at high risk for developing PTLD, being possible candidates for pre-emptive therapy, could be identified at an early stage. A recently developed quantitative EBV PCR was used that required only small amounts of whole blood, which is particularly useful for studies in children.
Patients and methods

In the Netherlands all pediatric LTx are performed in the University Medical Center Groningen. Approval was obtained from the Medical Ethical Committee of this hospital. All pediatric patients transplanted between January 1, 2000 and December 31, 2001 and surviving the first 6 months after LTx were included in the study. EBV serology was routinely performed before LTx (immunofluorescence assay based on IGM antibodies against virus capsid antigen and IgG antibodies against virus capsid antigen, early antigen and against Epstein-Barr nuclear antigen-1 [EBNA-1]).

In the study period, 13 pediatric patients were included. Table 1 shows clinical details of the included patients. The mean age was 5.7 years (range 0.6-16). Six children were under 4 years of age. The indications for LTx were biliary atresia (n=6), autoimmune hepatitis (n=2), Alagille’s syndrome (n=1), primary sclerosing cholangitis (n=1), alpha-1-antitrypsin deficiency (n=1), cryptogenic liver fibrosis (in combination with end-stage renal failure; combined liver-kidney transplantation, n=1), and hepatic metastasis of insulinoma (n=1). In 4 patients a retransplantation was needed. In total 8 full-size liver grafts were used and 9 partial grafts. The mean cold ischemia time was 8.9 h (range 5.6-12.5 h) and the mean anhepatic phase was 109 min (range 65-225 min). The age of the donors varied between 2 and 58 years, with 60% over 20 years.

The immunosuppressive regimen consisted of tacrolimus and prednisolone in 10 of the 13 patients. During the first 3 months after LTx trough levels of tacrolimus were aimed at 10–15 µg/l, and at 5–10 µg/l thereafter. Prednisolone was started at a dose of 1 mg/kg at day 1 after LTx and was tapered to a dose of 0.2 mg/kg/day at 3 months. Patients with autoimmune hepatitis or primary sclerosing cholangitis received a cyclosporine-

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Table 1. Demographic data of the included patients.
based immunosuppressive regimen aiming for trough levels at 200 – 250 μg/l the first 4 weeks and at 100-150 μg/l thereafter. Biopsy-proven acute rejection was treated with intravenous methylprednisolone (20 mg/kg) for three days. The follow-up for clinical signs of PTLD ended June 30, 2003 (mean follow-up 32 months).

All patients received oral acyclovir during the first 4 weeks after LTx. If a cytomegalovirus (CMV)-negative recipient received a CMV-positive graft; oral ganciclovir was administered after day 10 for 3 months after LTx. Upon indication of CMV infection as inferred from quantitative determination of pp65-antigen in peripheral blood, intravenous ganciclovir or foscarnet was started until pp65-antigen levels dropped below limit of detection. Oral ganciclovir was then restarted for an additional period of 3 months.

Blood samples were taken from every patient included in the study at monthly intervals after LTx. Blood samples for EBV PCR assay were stored in NASBA buffer at −80 °C as described previously in blinded fashion until analysis. The clinicians involved in the patients’ care were not informed about the test results for the first half year after LTx. Thus, the EBV PCR values did not lead to alteration of immunosuppression during the study period. Biochemical values were determined at identical time points as EBV PCR sampling. Clinical information was obtained from outpatient clinic records and from diaries that each patient held.

Quantitative competitive EBV polymerase chain reaction (PCR) was performed as described previously. In short, the DNA equivalent of 5 μl of whole blood was amplified in a qualitative EBNA-1 PCR, using the QP1/QP2 primer pair. Negative samples were subsequently tested by beta-globin PCR to check for DNA quality and sample inhibition, and if negative, DNA isolation was repeated and qualitative PCR was performed on the new isolated sample. The EBV DNA load in positive samples was subsequently determined by quantitative competitive EBNA-1 PCR (QP1/QP2 primer pair). As internal control for accuracy and reproducibility of quantification, a fixed amount of calibrator plasmid DNA, containing an internally randomized sequence of 23 nucleotides, was co-amplified in each experiment in duplicate. In addition, all samples were screened blindly, and appropriate negative and positive controls for DNA isolation, preparation of PCR master mix and enzyme immune assay detection were included (1 negative control for each 10 tested samples and 1 positive control per experiment).

An EBV infection was defined by a positive EBV PCR above the detection limit of 2000 copies/ml. A primary EBV infection was defined by a positive EBV PCR in a patient with negative EBV serology prior to LTx. EBV reactivation was defined as a positive EBV PCR in a patient with a positive EBV serology prior to LTx.

Results

Figure 1 and Table 2 show the results of EBV serology before LTx and the development of infection and PTLD during the 2-year study period in the 13 patients. As could be expected in the age group studied, the majority of patients (9 of 13, 69 %) was EBV seronegative at the time of LTx. One patient died 7 months after LTx on
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the waiting list for retransplantation due to biliary cirrhosis, attributed to ischemic type of biliary lesions.

EBV DNA in seropositive patients
In one of the four patients that were EBV seropositive before LTx, EBV PCR was found positive at 4 time points after LTx (Fig. 2). In this patient, the first positive EBV PCR was found at 11 days after LTx. The peak value of EBV PCR, at 5 months after LTx, was relatively low, less than twofold the lower level of detection (3,600 copies/ml). The patient passed an EBV infection just a month before LTx, with clinical signs of fever and lymphadenopathy, as confirmed by a positive IgG and IgM antibodies for EBV viral capsid antigen on the day of LTx. There were neither clinical signs nor biochemical indications of EBV reactivation after LTx. None of the other three patients who were EBV seropositive before LTx developed a positive EBV PCR during the observation period.

EBV DNA in seronegative patients
Nine patients were EBV seronegative before LTx. The EBV status of the donor organ was not routinely tested, but the age of the donors was over 20 years in 60%. It is well known that 90% of persons at 20 years of age have undergone an EBV infection. The likely presence of EBV in the donor organs was confirmed by the development of positive EBV PCR in most of these 9 patients in the first 6 months after LTx. Eight patients passed a primary infection after LTx based on an EBV viral load that was at least above the threshold of detection. The primary infection was confirmed by seroconversion a half year after LTx. Two of these 8 patients who underwent a primary EBV infection developed a PTLD. The longitudinal biochemical and viral parameters will be discussed below for the patients who passed a primary EBV infection and for each of the PTLD patients.

Figure 1. Epstein-Barr virus (EBV) serology status before liver transplantation (LTx) and EBV PCR results of the study group after LTx.
Table 2. Patient data concerning age, EBV serology before and after LTX, and details of EBV infection after LTx.

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<th>First EBV + in days after LTx</th>
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Patients with a primary infection, who did not develop a PTLD

Six patients underwent a primary infection after LTx, but did not develop a PTLD. Figure 2 shows the EBV results in one of these patients. None of the patients, however, developed clinical symptoms related to a primary infection or any elevated liver enzymes that could be related to the elevated EBV PCR. The mean peak viral load in the fist half year after LTx in this group was 16,600 copies per ml (SD 13,800). The highest peak viral load varied in the six patients between 3550 and 38,890 copies/ml. All patients did well during follow-up of at least 20 months after LTx. Until June 2003 none of these patients had any clinical signs that could be attributed to a PTLD.

Four of these six patients developed an acute rejection (all grade 2, 2 in the first week and 2 at 4 months after LTx), which was treated successfully with methylprednisolone intravenously. A high EBV PCR was found only in one patient at the time of rejection. The liver biopsy of this patient was consistent with rejection and not with viral hepatitis, and normalized after methylprednisolone treatment.

Patients with a primary infection who developed PTLD

Two of the total 13 included patients developed a PTLD. PTLD was diagnosed at 4 months and at 20 months after LTx. Figure 2 shows the longitudinal data of EBV PCR levels of the first patient. This patient, 15 years old at LTx, developed fever, a lympho-

Figure 2. Circulating EBV load in four patients in the first half year after liver transplantation (LTx). Horizontal axis: days after LTX, vertical axis: EBV PCR in copies/ml, logarithmic scale.
ma in the cervical region, and diarrhoea at 4 months after LTx. The lymphoma was surgically removed for histological analysis. Histological samples were also obtained from a colonic ulcer that was found during colonoscopy. Pathology of both lesions showed a monomorphic monoclonal PTLD. The patient was treated with reduction of immunosuppressive medication and intravenous ganciclovir. Since clinical signs did not resolve, patient was subsequently treated with four courses of monoclonal anti-CD 20 antibodies (rituximab, 375 mg/m²) after which the clinical symptoms resolved. Follow-up computed tomographic (CT) imaging and colonoscopy no longer showed presence of PTLD lesions. This patient developed a biliary cirrhosis, however, probably secondary to ischemic type of biliary lesions, as demonstrated by ERCP. Due to end stage liver failure the patient died while on the waiting list for a retransplantation 4 months later. At 52 days after LTx, EBV PCR became positive for the first time. The maximum level of EBV copies was 20,500 copies/ml at 4 months, when PTLD was diagnosed. Reduction of immune suppression caused a rapid decline of the EBV viral load in blood, even until below the limit of detection before rituximab treatment was initiated. Interestingly, however, the symptoms of fever and lymphadenopathy remained present at this stage, and only disappeared after rituximab treatment.

The second patient who developed PTLD was 3 years old at LTx. This patient had clinical symptoms of fatigue and fever starting 3 months after LTx. Figure 2 shows the longitudinal data of EBV PCR in this patient. The first EBV PCR positive sample was found at 44 days after LTx. The maximum EBV load was 446,000 copies/ml at 119 days after LTx. About 3 months after LTx liver enzymes increased and a liver biopsy was performed. Histology showed indications for a viral hepatitis. An in situ hybridisation of EBV-encoded mRNA-1 (EBER-1) to detect EBV infected B lymphocytes was negative. However the liver histology, in combination with a high circulating EBV viral load, led to the suspicion of active EBV infection, although this could not be confirmed by the EBER-1 staining. Immunosuppression was reduced, what resulted in biopsy-proven resolution of the hepatitis at twelve months after LTx. The presenting symptoms of fatigue and fever seemed to improve but did not completely resolve. Also, the circulating viral load and the liver enzymes remained elevated. At 18 months, the patient developed diarrhea. Colonoscopy did not result in an explanation for the diarrhea, neither macroscopically, nor in colon biopsies. CT studies of abdomen and thorax did not show lymphomas. Since a PTLD was nevertheless suspected, tonsillectomy was performed at 20 months after LTx, which showed hyperplasia of polymorphic EBER-positive, B lymphocytes without abnormalities of tissue architecture. These histological features can be interpreted as a pre-PTLD form, but this form has also been accepted as part of the continuum of EBV disease, and included under PTLD. As stated above, the clinical symptoms persisted and even increased during reduction of immunosuppression. Therefore, rituximab treatment was initiated, which cleared the symptoms.

Both patients had undergone an acute cellular rejection shortly after LTx. Patient 1 had in the first week, a grade 2 rejection which responded well to methylprednisolone treatment. Patient 2 had a grade 3 rejection 2 months after LTx. At the time of this rejection period a high EBV PCR was found; however, the histopatho-
logical findings were consistent with the diagnosis of rejection and responded well with methylprednisolone treatment. At 3.5 month after LTx, a liver biopsy specimen showed atypical inflammation, probable compatible with a primary Epstein Barr virus infection.

Figure 3 shows the peak viral loads of all the patients with positive EBV PCR. The highest peak viral load was seen in the patient with clinical symptoms of a primary infection, who developed a PTLD after LTx. Both PTLD patients had high circulating EBV loads during the first half year after LTx, but similar values were found in patients with a primary infection. In the patient with a reactivation of EBV infection the peak viral load was just above detection level. EBV serology a half year after LTx showed positive EBV IgG antibodies against EBV viral capsid antigen in all patients who passed a primary infection. In neither patient, however, antibodies against EBV nuclear antibodies were found. In each patient with a primary EBV infection EBV PCR became first positive between 30 and 90 days (mean at 64±20 days) after LTx. In the two PTLD patients the first detectable viral load was found at 44 and 54 days after LTx, respectively.

Five of the eight patients with a primary EBV infection received ganciclovir for 3 months after LTx. In four patients ganciclovir was used as prophylactic treatment against primary CMV infection. One patient had received ganciclovir intravenously for 2 weeks as treatment for a reactivation of CMV infection, followed by oral ganciclovir treatment for 3 months. In the patients who used oral ganciclovir, EBV PCR become first positive at 61±20 days after LTx (n=4). Patients that had not received ganciclovir treatment were EBV PCR positive at 76±18 days after LTx (n=3). The peak viral load was 21,900±14,000 copies/ml in the patients, who used ganciclovir and 15,700±15,800 copies/ml in the group who did not. Thus, in this small cohort of pediatric liver transplant patients, pre-emptive treatment with ganciclovir did not lead to a retardation of the primary EBV infection or a lower peak DNA load.
Discussion

We investigated the relationship between EBV DNA load in peripheral whole blood and primary EBV infection and the development of PTLD. The majority of the included patients underwent a primary EBV infection after LTx. In these children, the circulating EBV DNA load could be used as a parameter to follow the EBV infection. The circulating EBV DNA load was higher in the patients with a primary infection than in patients who were EBV seropositive prior to LTx. Two patients who developed a PTLD had a high circulating viral load, but nevertheless they could not have been reliably identified on the basis of preceding EBV DNA load levels. Most of the patients did not have clinical symptoms or elevated liver enzymes that could be related to elevated EBV DNA load. The two patients, who had clinical symptoms related to an EBV infection, developed a PTLD.

The relation between high circulating EBV DNA load and a primary EBV infection after LTx was previously described by Savoie et al.\(^9\) In pediatric patients with a primary EBV infection after LTx a higher amount of EBV infected lymphocytes was seen compared with patients who were seropositive before LTx, using lymphocyte cultures. One patient with PTLD was described, who had the highest number of infected lymphocytes. Smets et al.\(^15\) concluded that patients with a primary infection and high EBV DNA load were at risk for developing a PTLD, but could not identify the patients that developed a PTLD based on the quantitative EBV PCR results alone, similar to our present findings. A high amount of EBV primary infection in pediatric patients after LTx with a limited number of clinical symptoms was also observed by Smets et al.\(^16\) and Spada et al.\(^17\) Smets et al. described that 80% of all patients who were EBV seronegative before LTx underwent a primary EBV infection in the first months post LTx using a qualitative EBV PCR. Only four patients had clinical symptoms and all developed a PTLD, but also one of the patients without symptoms developed a PTLD. Spada\(^17\) reported a cohort of 100 pediatric patients, who had been EBV seronegative prior to LTx. Only 12 children developed an EBV infection with clinical symptoms, and one of these a PTLD. Similar to what we have seen, the combination of clinical symptoms and a primary EBV infection seems particularly relevant for the development of a PTLD.

PTLD has been associated with high EBV DNA load in the peripheral blood.\(^9\) Quantitative PCR techniques have been used as a method for early detection and for evaluation of treatment of PTLD.\(^9;18;19\) Our present data indicate, however, that similarly elevated EBV viral loads are observed in patients with a primary EBV infection without clinical symptoms, with a normal function of the liver graft, and without the subsequent development of PTLD during follow-up period of 2 years. It is tempting to speculate that the inhibited T cell function is just enough to prevent (completely) uncontrolled B cell proliferation. The present results therefore imply that quantitative EBV PCR does identify a group of patients at increased risk for developing a PTLD, but that it lacks the sensitivity to identify individual patients actually developing a PTLD in an early stage. It can not be excluded that the simultaneous quantification of EBV specific T-cell function\(^15\) could increase the specificity to detect PTLD. Unfortunately, presently described methodologies for determination of EBV specific T cell
function are very laborious and not (yet) available for routine clinical application. In this study, we used a competitive quantitative EBV PCR for a prospective follow-up of serial EBV DNA load determination in pediatric patients after LTx. We do realize that the number of patients is relatively small. Yet the present approach allowed for the follow-up of all patients, whereas in other studies a transectional design was applied or only selected patients were followed. The study design to sample every 4 weeks might not have been frequent enough, and high peaks might have been missed. A very short doubling time in a patient with a primary infection was shown previously. A sampling frequency of every week during primary EBV infection might be more appropriate, but this is difficult to effectuate in an out-patient setting. Histological material was gathered on indication of clinical symptoms, and not on the basis of an elevated EBV DNA load or primary infection. Nevertheless, it is not likely that we have missed a clinically relevant PTLD considering the clinical history of these other patients.

In three patients with a primary infection we have seen an elevation of liver enzymes after the period of the first 2 weeks post LTx, attributed to rejection based on the biopsy result. All were treated with methylprednisolone. Retrospectively we found in two patients a high EBV PCR result at the same time. In one of these patients hepatitis attributed to an EBV infection developed 1 month later. In retrospect, the events are compatible with the possibility that the treated rejection period might in fact have been a first presentation of EBV hepatitis. The differential diagnosis of EBV hepatitis and rejection can be difficult. In a study of liver biopsies in patients with PTLD, a combination of rejection and hepatitis was seen in 80%, and in only two-thirds an EBV staining was positive. Our finding underline that the diagnosis of rejection should be made with caution.

The initial treatment of PTLD involves reduction of immunosuppression. If this approach is not successful, anti-B cell (CD20) monoclonal antibodies have been used, as we did in our patients. The role of antiviral medication, immunoglobulins, chemotherapy and experimental treatments such as infusion of cytotoxic T cells is not yet clear. More recently, pre-emptive treatment in patients at increased risk has been advocated. Mc Diarmid et al. treated all patients with EBV negative serology prior to LTx with the antiviral medication ganciclovir after LTx. Our data do not support a protective effect of ganciclovir on the development of a primary EBV infection. Oral ganciclovir treatment for the first three months post LTx did not prevent or retard primary infection, nor did it affect the peak height of EBV copy number in peripheral blood. Oral ganciclovir may not be absorbed sufficiently to reach clinically relevant levels. Alternatively, lytic viral replication may be not important in the pathophysiology of post-LTx EBV infection. Another approach of pre-emptive therapy involves reduction of immuno suppressive therapy in risk groups identified by a high EBV DNA, such as in patients with a primary infection. Mc Diarmid demonstrated that this strategy decreased the incidence of PTLD pediatric patients after LTx with 10 to 5%. The risk of reduction of immunosuppression is rejection of the transplanted graft. It has been shown however, that reduction of immune suppression when there is a high EBV DNA load can be done without rejection or with re-
jection in only a small percentage of patients. Based on these observations, a high EBV PCR could reflect an over-immunosuppressed condition, in which reduction of immunosuppression could re-establish the T cell control function and prevent PTLD. If we would have used a pre-emptive approach in our patients, such as reduction of immunosuppression, a cut-off level of 20,000 copies/ml would have included all PTLD patients but also three patients who spontaneously recovered from the primary infection without signs of PTLD.

In conclusion, our data show that serial EBV DNA load in pediatric liver transplant recipients allow identification and monitoring of primary EBV infection. Clinical symptoms during a primary EBV infection could indicate patients at risk for developing a PTLD. Elevated EBV DNA loads without clinical symptoms, however, do not seem specific for the development of PTLD.

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