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Epstein-Barr virus DNA in serum after liver transplantation – surveillance of viral activity during treatment with different immunosuppressive agents

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Abstract In immunocompromised HIV-infected and transplanted patients, there is a risk of developing Epstein-Barr virus (EBV)-associated lymphoproliferative disorders (LPD) and lymphomas. EBV has previously been detected by the polymerase chain reaction (PCR) in cerebrospinal fluid from all AIDS patients with EBV-associated cerebral lymphomas. We therefore thought it would be of interest to determine whether transplant patients with extracerebral EBV-associated LPD have detectable EBV genomes in serum. Nested PCR (nPCR) showed that 58 % (18/31) of liver transplant (LTX) patients had EBV DNA in 17 % (21/125) of serum samples obtained within the first 3 months after LTX. In 39 % (7/18) of the patients, the first EBV nPCR-positive sample was found within 2 weeks post-LTX. Basic immunosuppression with cyclosporin A or FK506 did not seem to influence the frequency of detectable EBV genomes in serum. In contrast, positive EBV nPCR correlated to secondary OKT3 treatment for severe acute rejection ($P = 0.009$). EBV-associated malignant lymphoma developed in three patients 2–6 months post-LTX. In all of them, EBV DNA was amplifiable within 12–14 days after LTX. The EBV antibody titers were not directly related to detectable EBV DNA in serum. We conclude that monitoring of LTX patients receiving increased immunosuppression by nPCR for EBV DNA in serum may help in the early identification of those at risk of developing EBV-associated LPD.

Key words Epstein-Barr virus, liver transplantation, immunosuppression · Liver transplantation, Epstein-Barr virus, immunosuppression · virus DNA, liver transplantation

Introduction

Primary Epstein-Barr virus (EBV) infection may be asymptomatic or may cause infectious mononucleosis of varying severity in healthy subjects. Progressive multiorgan lymphoproliferative disorders (LPD) and lymphomas may occur in HIV-infected or transplanted patients with compromised immunity [6, 9, 22]. Immunosuppression is a prerequisite for the development of EBV-associated LPD [21]. The risk is considerable when drugs that inhibit specific T-cell cytotoxicity, such as cyclosporin A (CyA) and FK506, are given in cumulative doses [4]. Another risk factor is T-cell depletion of donor marrow at the time of bone marrow transplantation [26]. In the transplanted subset of patients, the clinical effects of EBV may be difficult to distinguish from conditions due to other herpes viruses, such as cytomegalovirus (CMV) and human herpes virus 6 (HHV-6). The lymphocytic infiltration in EBV hepatitis may resemble that found during graft rejection in liver transplant recipients [33].

EBV mononucleosis is considered to be an immune-mediated disease that occurs when cytotoxic T cells at-

Table 1 Indications for liver transplantation (LTX), demography, and perioperative blood transfusions in 31 patients

LTX indication	*n*	M/F	Age (years)	Blood transfusions (units)
Acute liver failure	2	0/2	10, 58	24.5
Chronic liver disease	27	15/12	range 1–63	14.0
Liver malignancy	2	0/2	18, 26	4.5
Total	31	15/16	38.5 (median)	14.0 (median)

Table 2 Patient groups according to basic immunosuppression, rejection treatment, and number of patients treated for acute rejection

Group	Patients *(n)*	Basic immunosuppression	Rejection treatment	Patients treated for acute rejection *(n)*
A	7	CyA[a] + AZA + steroids	High-dose steroids (recycling)	7
B	12	CyA[a] + AZA + steroids	High-dose steroids + OKT3[b]	12
C	8	FK506[c] + steroids	High-dose steroids (recycling)	7
D	4	FK506[c] + steroids	High-dose steroids + OKT3[b]	4

[a] Cyclosporin A, 10 mg/kg per day orally or 3 mg/kg per day i.v., given in combination with azathioprine, 1.5 mg/kg per day i.v. followed by 1–2 mg/kg per day orally, and steroids (prednisolone), which was tapered from 200 to 20 mg/day over a period of 6 days

[b] OKT3 (monoclonal anti-CD3 antibody; Ortho Pharmaceuticals, N.J., USA), 5 mg/kg per day given i.v. for 7–10 days

[c] FK506, given in doses of 0.15 mg/kg per day i.v., followed by 0.3 mg/kg orally

tempt to gain control over transformed B lymphocytes [7, 31]. Although EBV is easily detected in leukocyte fractions of peripheral blood during infectious mononucleosis in immunocompetent individuals, less than one-third (27 %) of the serum samples drawn during active disease have been shown to contain virus when examined with the polymerase chain reaction (PCR) [10]. The EBV nPCR negativity in serum is probably due to the lack of viral replication and virion production in infected B cells [10, 24]. Other lymphotropic herpes viruses, such as CMV and HHV-6, are more frequently detectable in serum during active disease [3, 20, 27]. EBV nPCR was found in cerebrospinal fluid from only 50 % of patients with neurological symptoms in connection with primary EBV infection (unpublished observation). The same method, however, detects EBV in cerebrospinal fluid from all AIDS patients with EBV-associated cerebral lymphoma [5]. It was therefore of interest to determine whether transplanted patients with EBV-associated LPD in localizations other than the central nervous system have detectable EBV genomes in serum.

In this study, we assayed the presence of EBV genomes using the nested polymerase chain reaction (nPCR) in serum obtained from liver transplant recipients who were on different immunosuppressive regimes. The samples were obtained during 3 months after liver transplantation (LTX). The serological response to EBV was evaluated in the same serum samples. Three patients developed EBV-associated lymphomas after transplantation.

Materials and methods

Patients, immunosuppression, and antiviral therapy

Thirty-one patients undergoing orthotopic LTX between October 1990 and August 1994 were prospectively monitored for the first 3 post-LTX months. The median clinical follow-up period was 4.5 years (range 2 months–5 years). All of the patients gave their informed consent prior to inclusion in the study. The study was approved by the local ethics committee at Huddinge Hospital. The demography of the patients, the indications for LTX, and the number of perioperative blood transfusions are presented in Table 1. The serum samples were collected before LTX, at 2 weeks post-LTX, and at 1 and 3 months post-LTX. Ninety-seven percent (30/31) of the pretransplant serum samples were available and of good quality, whereas 90 % (83/92) of the post-LTX samples were available. Additional samples were obtained from a few patients on other occasions within 3 months of LTX. Thus, the total number of serum samples studied was 125. The samples were stored at – 70 °C for the retrospective analyses.

The patients were divided into four groups based on the different types of immunosuppression, which consisted of cyclosporin A (CyA; Sandoz, Basel, Switzerland) in combination with azathioprine (AZA) and steroids (groups A and B) or FK506 (tacrolimus; Fujisawa, Germany), instead of CyA, and steroids (groups C and D) (Table 2). Episodes of acute rejection were treated with 1 g methylprednisolone sodium succinate, followed by tapering of the prednisolone dose over 6 days (steroid recycling). Patients in groups B and D suffered from episodes of steroid-resistant rejections and were further treated with the monoclonal anti-CD3 antibody OKT3 (Ortho Pharmaceuticals, N.J., USA) for 7–10 days.

Seven patients were included in a randomized, placebo-controlled study of high-dose acyclovir, 3200 mg daily (Wellcome, London, UK), given as prophylaxis against CMV infection/disease during the first 3 post-LTX months. Three of these patients received acyclovir in the study (the other four the placebo); one addi-

Table 3 Complications in patients with or without detectable EBV DNA in serum within 3 months after liver transplantation (LTX)

Complications after LTX	EBV DNA-negative patients		EBV DNA-positive patients		P^a
	(*n*)	[%]	(*n*)	[%]	
Acute rejection	5	38	12	67	0.12
CMV infection	3	23	4	22	0.22
CMV disease	3	23	8	44	
Septicemia/candidemia	2	15	4	22	0.50
EBV lymphoma	0	0	3	17	
Death ≤ 1 year post-LTX	1	8	4	22	0.04
Total number of patients at risk	13		18		

[a] According to Fisher's exact test

tional pediatric liver recipient was given acyclovir prophylaxis outside the study.

Epstein-Barr virus serology and genome analyses

Epstein-Barr virus IgG and IgM antibodies to the replicative antigens in P3RH1 cells (mainly the virus capsid antigen, VCA) and to EBV nuclear antigen (EBNA) were determined by immunofluorescence, according to published standard methods [14]. Activated NC37 cells were fixed in acetone or methanol and were used to detect early antigen EA R + D and EA R antibodies, respectively. IgM and IgG against the EBNA1 antigen were determined in a peptide ELISA with the p107 peptide, representing the glycine-alanine repeat of EBNA1, as the antigen [15]. Reduction of IgG and IgM p107 titers was defined as a difference in optical density of 0.5 or more between the antibody titers of samples obtained pre-LTX and 3 months post-LTX, respectively. In addition, serological markers of CMV, herpes simplex virus, and varicella zoster virus were analyzed pre-LTX, as well as 1 and 3 months post-LTX.

EBV DNA genomes were normally extracted from serum; however, if this was not available, plasma was used. The samples were centrifuged at 500 g for 10 min, according to the routines at our laboratory. The QIA amp blood kit (Qiagen, Hilden, Germany) was used in accordance with the instructions of the manufacturer, with one exception. While 200 µl of serum was added to the column as recommended, only 50 µl of H_2O was used for elution in order to concentrate the DNA. However, in 22 of the 125 samples extracted (18 %), only 50-µl serum was available. The EBV nPCR was performed on duplicates of 10-µl sets of extracted DNA on two occasions. Examination in duplicate is necessary. The examination should be repeated if only one of two is positive because one may be contaminated, despite rigorous precautions against contamination. Since extracted DNA was used in the study, we regarded the risk of inhibition by the serum preparations as very small.

An nPCR amplifying a 147 bp-long fragment of the BAM HI 1 gene was used, as previously reported [5]. The EBV nPCR was considered positive when EBV DNA could be amplified in three of four sample sets. In each nPCR examination, three dilutions of P3HR1-infected cells that had been repeatedly examined were included as positive controls. The last dilution was a borderline control and no assay was accepted unless the borderline control was positive.

EBV-associated LPD and lymphoma were diagnosed by immunohistological examination of a fine-needle aspiration biopsy or of core biopsy material from the tumors using specific monoclonal antibodies against tumor cell lines and showing EBV nuclear antigen (EBNA) and/or EBV genome in the tumor cells [1, 19].

The patients' files were carefully studied to compare the results of the virological analyses with the clinical data. Evaluation of the degree of acute liver graft rejection in liver core biopsy specimens was performed according to classification of Snover et al. [29] with grades 0–4 (Table 3). This table also presents patients in each study group with post-LTX complications including EBV-associated lymphoma.

Statistical methods

Fisher's exact test and the Wilcoxon Mann-Whitney test were used.

Results

EBV nPCR

Nested PCR revealed that 18 of 31 patients (58 %) were EBV DNA-positive in one or more samples within the first 3 post-transplant months (21/125, or 17 %, of the samples). In six of the nPCR-positive patients, EBV DNA could be amplified even in the pre-LTX samples. In seven other patients, the first EBV nPCR positivity was found within the first 2 weeks after LTX. In 11 of 31 patients, EBV DNA was also amplified in one or two of four sample sets. In 9 of 11 patients, this sample was obtained before or after the sample with the most intense nPCR (in three or more of four sets).

EBV DNA was amplified in 1 of 22 (4.5 %) 50-µl serum samples. This can be compared to 20 out of 103 (19 %) PCR-positive 200-µl samples ($P = 0.36$, Fisher's exact test). The one positive EBV nPCR in a 50-µl sample was preceded by a positive PCR in a 200-µl sample.

EBV DNA was detected as frequently during the first 3 post-LTX months in patients who received high-dose acyclovir prophylaxis against CMV as in patients randomized into the placebo group.

EBV nPCR in relation of graft rejection

Twelve of 18 patients (67 %) with EBV nPCR positivity suffered from acute rejection of grade 2 or more, according to Snover's classification [29]. Five of 13 EBV nPCR-negative patients (38 %) were also diagnosed as

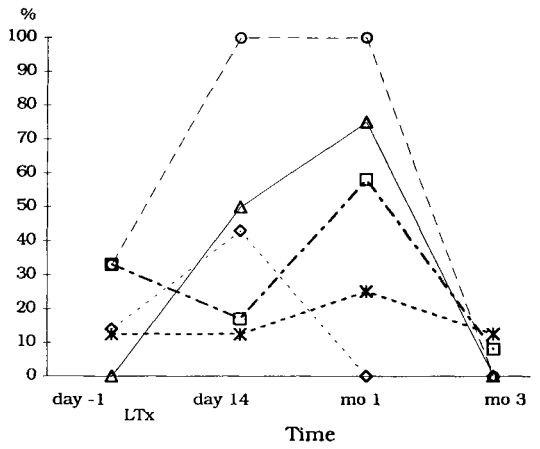

Fig. 1 EBV DNA detected by nested PCR in serum from liver recipients before and after liver transplantation (LTX). Values represent percentage of positive samples analyzed. Four types of immunosuppressive therapy were used: cyclosporin A (CyA) + azathioprine (AZA) + steroids (S) without *(A)* and with *(B)* OKT3, and FK506 + steroids (S) without *(C)* and with *(D)* OKT3. The findings in three patients with EBV-associated lymphoma *(E)* are shown separately. --◇-- A CyA + S + AZA; --□-- B CyA + S + AZA + OKT3; --✳-- C FK506 + S; --△-- D FK506 + S + OKT3; --○-- E Lymphoma pts

having at least a grade 2 acute rejection ($P = 0.12$, Fisher's exact test). The patients with severe rejections were initially treated with high doses of steroids; in addition, they required OKT3 and/or FK506 to resolve the rejection episodes.

EBV nPCR in relation to immunosuppression

EBV DNA was detected with nPCR in 12 out of 19 (63 %) patients who were treated with CyA as basic immunosuppression (groups A and B). EBV DNA was amplified in 6 out of 12 (50 %) patients who received FK506 as basic immunosuppression (groups C and D; Fig. 1). Thus, the basic immunosuppression did not seem to influence the frequency of detectable EBV genomes ($P = 0.36$, Fisher's exact test). In contrast, EBV DNA was found in 13 out of 16 (81 %) patients who needed OKT3 for treatment of severe episodes of acute rejection (groups B and D). In 5 of 15 (33 %) EBV DNA-positive patients, no treatment other than high doses of steroids was needed for the rejection episodes (groups A and C; $P = 0.009$, Fisher's exact test).

EBV nPCR in relation to lymphomas

Three patients developed EBV-associated B-cell lymphoma of non-Hodgkin type 2–6 months after LTX. The EBV genome was amplifiable in all three cases in the samples obtained 12–14 days post-LTX (Fig. 1). In addition, one case showed EBV nPCR positivity in the pre-LTX sample. This patient had been examined for lymphadenopathy in her left axilla 6 months earlier without a conclusive diagnosis. Two months post-LTX, an EBV lymphoma developed lateral to her breast. The other two patients were found to have lymph node enlargement in the neck, and in the lungs/intra-abdominally 5 weeks and 6 months after LTX, respectively. Two of these patients, who developed lymphomas, were treated with OKT3 for a steroid-resistant acute rejection.

EBV serology

Sixteen of 18 patients with detectable EBV DNA genomes were evaluated for EBV VCA IgG, p107 IgM, p107 IgG, and the p107 IgG/IgM in samples obtained at the same time as the first nPCR positivity (range 4 days–3 months post-LTX). When the serological titers in patients with detectable EBV DNA in serum were compared with those obtained 3 months post-LTX from 11 out of 13 patients without amplifiable EBV DNA, there were no significant differences ($P = 0.47$, $P = 0.49$, $P = 0.48$, and $P = 0.47$, respectively, Wilcoxon Mann-Whitney test). Nor were there any differences when the results of the antibody tests performed at 3 months in both patient groups were compared with each other ($P = 0.46$, $P = 0.49$, $P = 0.48$, and $P = 0.48$, respectively, Wilcoxon Mann-Whitney test). However, 12 of the 15 patients (80 %) with EBV DNA in serum showed a reduction in the IgG p107 titer between 1 and 3 months post-LTX. This was significantly more often than in 4 out of 11 patients (36 %) without EBV DNA in serum ($P = 0.03$, Fisher's exact test).

One of the patients had IgM to VCA (1:160) at 3 months post-LTX. However, EBV DNA was not amplified in any of the serum samples from this patient who suffered from primary CMV hepatitis during the same period. Antibodies against the different components of EBV early antigen, EA D, (1:20 to 1:80) were found pre-LTX in three pediatric patients and in one young adult liver recipient. The latter patient continued to show EA D during the 3-month study period, while the EBV nPCR was negative.

Patient survival

The main infectious complications are presented in Table 3. We found no significant difference in the occurrence of CMV infection and/or disease, septicemia and/or candidemia in the patients with detectable EBV DNA as compared to those without EBV DNA during the 3 post-LTX months.

The 1-year survival rate was 84 % (26/31 patients). EBV DNA was present in 4 out of 5 patients who died within the 1st post-LTX year. This was significantly more than in those who survived ($P = 0.04$, Fisher's exact test). Two of the three patients with detectable EBV DNA in serum who developed EBV-associated B-cell lymphomas survived after the immunosuppression was reduced and satisfactory chemotherapy was given. The third patient had a fatal outcome with septicemia and malignant lymphoma 7 months post-LTX. Another patient died early, at 2 months post-LTX, with a reactivated EBV infection, CMV pneumonitis, and disseminated candidosis, combined with aplastic anemia. The remaining two patients died 7 and 8 months post-LTX, respectively, without any clear evidence of EBV nPCR positivity.

Discussion

Epstein-Barr virus is widely spread in all populations; it has a prevalence of 95 % or more [11]. It is transmitted to most healthy subjects without apparent disease, but in immunocompromised patients there is a risk of severe infectious mononucleosis and LPD. We found that 58 % of our LTX patients (18/31) showed EBV activation, measured as EBV DNA in serum using nPCR, during 3 post-LTX months. In most analyses, DNA was concentrated from 200-µl serum to 50 µl of elution buffer before nPCR. On some occasions, only 50-µl serum was available. However, nPCR did not reveal EBV DNA significantly more often in 200-µl serum samples than in 50-µl samples. The EBV DNA finding was significantly correlated to OKT3 treatment of severe episodes of acute rejection. Three patients were diagnosed as developing EBV-associated lymphomas.

EBV nPCR positivity pre-LTX might be explained in one pediatric patient by a recent primary infection and in four others by the impairment of the host immune defense due to chronic liver disease [11]. Two of these patients suffered from hepatitis B- and hepatitis C-associated uncompensated cirrhosis, respectively. One patient had received cytotoxic treatment against malignant melanoma for 6 months pre-LTX. The fourth patient had had an unexplained tumor beside her left axilla that developed into a malignant EBV-associated lymphoma during 2 post-LTX months. EBV DNA was found sporadically in 11 patients and was repeatedly detected in 9 of 11 cases. This suggests that it is neccessary to monitor immunocompromised patients using EBV nPCR in serum in order to find those at risk of developing an active EBV infection. The immunosuppression should be tapered in patients who are repeatedly found to have EBV DNA in serum.

The basic immunosuppression did not seem to influence the frequency of detectable EBV genomes in serum. It has previously been reported that no disease could be attributed to EBV when FK506 was used as basic immunosuppression in liver recipients [28]. We found a correlation between EBV nPCR positivity and OKT3 treatment of severe episodes of acute liver graft rejection. This may have been due to specific cytolysis of host cytotoxic T cells [12, 17] by OKT3, allowing proliferation of EBV-infected B cells and activation with replication of EBV. Both European and U.S. multicenter trials have shown that primary use of FK506 significantly reduces the incidence of rejection and the need for OKT3. Thus lower cumulative doses of immunosuppressive drugs are required [8, 34]. Similarly, it has been reported that OKT3 did not increase the incidence of EBV infections when used as induction therapy instead of CyA, but it did so when used to treat acute rejections [18]. In addition, OKT3 antirejection treatment has been shown to be associated with an increased occurrence of post-transplant LPD [25, 32]. In our study, two of three patients with EBV-associated malignant lymphomas had received OKT3.

In this study, the EBV antibody titers showed no direct relation to EBV DNA detected in serum. Nor has serological surveillance of other herpes virus infections (CMV, HHV-6) proved to be of value in immunocompromised patients because of their failure to develop a significant humoral antibody response during an active viral infection [13, 16, 22, 23]. The immunosuppression may explain some undetectable EBV antibodies after LTX compared to the pre-LTX findings. The evaluation of antibody titers during the early post-LTX period is also complicated because large amounts of blood are usually transfused during LTX (mean 14 units of erythrocyte concentrate in this study). However, the EBV IgG p107 titer tended to be lower at the 3-month control after LTX than at the time when EBV DNA became detectable in patients with nPCR positivity, but not in patients without EBV DNA in the serum. The presence of EBNA1 antibodies, measured as antibodies against the p107 peptide, may be an indirect sign of T-cell control over EBV-transformed cells, and the low levels of p107 EBNA1 antibodies may reflect a defective T-cell control in the EBV nPCR-positive patients.

The 1-year patient survival rate of this LTX subgroup (84 %) is in line with previous reports of ours and from other centers [2, 35]. Two patients died of an EBV-associated infection – one of aplastic anemia together with septicemia and the other of LPD. In total, three patients suffered from LPD, and all of them showed EBV nPCR positivity within the 1st post-LTX month. Two of them had received increased immunosuppression against severe rejection with OTK3. Since post-transplant LPD and lymphoma are known to be associated with EBV, the first-line treatment should be a reduction in the maintenance immunosuppression [19, 30].

In conclusion, EBV DNA in serum appears to be a common sporadic finding in liver transplant patients (58%), but most patients showed no specific clinical symptoms. EBV genomes were found to be correlated to the secondary use of OKT3 in the treatment of severe acute graft rejection, independently of the use of CyA or FK506 as basic immunosuppression. Monitoring patients receiving increased doses of immunosuppressive drugs by nPCR for EBV DNA in serum may help in the early identification of those at risk of developing EBV-associated LPD.

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