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Polymerase chain reaction and in situ hybridization of Epstein-Barr virus in liver biopsy specimens facilitate the diagnosis of EBV hepatitis after liver transplantation

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Abstract A nested polymerase chain reaction (nPCR) for Epstein-Barr virus (EBV) DNA, RNA in situ hybridization (EBER-ISH), and immunostaining against the ZEBRA EBV protein for diagnosis of EBV hepatitis were performed on 43 liver biopsy specimens obtained from 18 patients in the 1st year after liver transplantation (LTX). The findings were related to liver histology and results of EBV-nPCR on concomitantly obtained serum samples. EBV DNA was detected in 30 % and RNA in 34 % of the liver biopsy specimens using nPCR and EBER-ISH, respectively, giving a significant correlation between the two methods ($P = 0.003$). All but one patient had detectable EBV DNA in serum samples obtained within 1 month of the biopsy. More than 90 % of the nPCR and EBER-ISH-positive biopsy specimens were obtained 3 months or less post-LTX. There was no significant difference in EBV genome findings in biopsy specimens with or without lymphocytic-immunoblastic infiltrates, either in nPCR ($P = 0.73$) or in ISH ($P = 0.73$). Two of three biopsy specimens with these histological changes suggesting a viral genesis were positive in EBV-nPCR but negative in ISH. Histopathological changes in EBV hepatitis may be nonspecific and masked by other complications. The use of EBV-nPCR and EBER-ISH in liver graft biopsy specimens of heavily immunosuppressed patients may give an early indication of EBV-related disease and can be used to guide therapeutic intervention.

Key words Epstein-Barr virus, hepatitis, liver transplantation · Polymerase chain reaction, hepatitis, liver transplantation · Liver transplantation, hepatitis, Epstein-Barr virus

Introduction

Epstein-Barr virus (EBV) is well known as the etiological agent of post-transplant lymphoproliferative disorders (PTLD) and B-cell type lymphomas in allograft recipients. In the liver-transplanted subset of graft recipients, th EBV-associated lymphoproliferative syndrome is found in up to 5 % of adult patients and in 15 % of pediatric patients [14, 16, 29, 32, 35, 42]. The type of immunosuppression, in particular polyclonal and monoclonal antilymphocyte antibodies such as ATG or OKT3 that cause defects in T-cell immune surveillance of the virus, is a known risk factor for the development of EBV-related disorders after transplantation [12, 19, 33, 38]. In less immunosuppressed transplant recipients, primary EBV infection or reactivation may manifest itself with severe mononucleosis-like symptoms [6, 7, 26]. EBV-associated hepatitis after liver transplantation (LTX) can induce graft dysfunction and may be difficult to diagnose, due to lack of specific blood chemistry markers. Concomitant histopathological features in a graft, such as signs of preservation injury and rejection, may also mask EBV infection [1, 17, 22, 24, 25, 31, 39, 40]. On the other hand, EBV is frequently present in lympho-

Table 1 *Occurrence of EBV DNA[a] and EBV RNA[b] using nPCR and in situ hybridization in liver graft biopsy specimens with various histopathological features with[c] or without immunoblastic-lymphocytic inflammation*

Histopathology of liver graft biopsy specimen	Time after liver transplantation							Total *(n)*	EBV DNA[a] *(n)*	EBV RNA[b] *(n)*	EBV-positive Total *(n)*
	Perioperatively *(n)*	Day			Month						
		3 *(n)*	10 *(n)*	28 *(n)*	2–3 *(n)*	6 *(n)*	12 *(n)*				
Normal							1 (1^{b})	1	0	1	1
Ischemia	9 (1^{b})		1 (1^{c})		1 ($1^{a,b,c}$)			11 (2^{c})	1	2	2
Nonspecific			4 ($1^{a,b}$)	1 (1^{c})	2 (2^{a}, $1^{b,c}$)			7 (2^{c})	3	2	3
Bile duct damage				1 ($1^{b,c}$)	1 ($1^{b,c}$)			2 (2^{c})	0	2	2
Acute cholangitis		1 (1^{c})	2 (2^{c}, $1^{a,b}$)	3 (3^{c}, $1^{a,b}$)			1 (1^{c})	7^{c}	2	2	2
Acute hepatitis					1 ($1^{a,c}$)			1^{c}	1	0	1
Acute rejection			8 (3^{a}, 2^{b})	3 ($1^{a,b}$)	1			12	4	3	4
mild			3 ($2^{a,b}$)	1	1			5	2	2	2
moderate			3	1				4	0	0	0
severe			2 (1^{a})	1 ($1^{a,b}$)				3	2	1	2
Chronic rejection					1 ($1^{a,b}$)	1 ($1^{a,b}$)		2	2	2	2
Total	9	1	15	8	7	1	2	43	13/43 (30%)	14/41 (34%)	17/41 (41%)

cytes, both in healthy and diseased persons. The pathogenetic role of EBV, when detected by sensitive methods in a blood-rich organ like the liver, is often difficult to establish. However, it is important to diagnose EBV-associated disease since the treatment is different for EBV-associated hepatitis and for liver graft rejection.

The aim of this study was to evaluate whether the detection of EBV DNA using the nested polymerase chain reaction (nPCR) on serum and in liver graft biopsy specimens, with or without the detection of EBV RNA, using in situ hybridization (ISH) in biopsy specimens, correlates with the histopathological featurs of EBV hepatitis (lymphocytic-immunoblastic infiltration) in liver transplant patients, as suggested by Alshak et al. [1]. The occurrence of EBV DNA and EBV RNA in liver biopsy specimens was compared to histopathological features in the same biopsy specimens in a double-blind fashion.

Materials and methods

Patients, immunosuppression, and antiviral therapy

The study population consisted of 18 liver recipients who underwent transplantation during the period 1990–1992. There were eight females and ten males with a median age of 26.5 years (range 1–59 years) and 34.5 years (range 7–63 years), respectively. Three females and two males were less than 16 years of age. There were 7 female and 11 male donors with a median age of 34.5 years (range 5–58 years) and 46.0 years (range 1–53 years), respectively. One female and one male donor were less than 16 years of age. All but one recipient were EBV-seropositive. The EBV serological status of the donors was not analyzed. However, given their median age, it can be assumed that the majority of them were EBV-seropositive.

The basic immunosuppressive therapy consisted of cyclosporin A (CyA; Sandimmun, Sandoz, Basel, Switzerland), azathioprine, and steroids in 12 patients and tacrolimus (Prograf, Fujisawa, Germany) combined with steroids in 6 patients. Episodes of acute rejection were treated with high-dose steroids (17 patients) and, if ineffective, with monoclonal antibodies against (CD3 lymphocytes (OKT3; Ortho Pharmaceuticals, N.J., USA) for 7–10 days (8 patients) [5]. In addition, OKT3 was given to one patient as induction therapy on days 1–3 post-LTX instead of CyA, due to renal failure. Thus, nine patients received OKT3 during the 1st post-LTX months.

Ganciclovir (Cymevene, Synthex Nordica, Södertälje, Sweden) was given to four patients against cytomegalovirus (CMV) infection or disease during the study period.

This study on EBV hepatitis was approved by the local Ethics Committee at Huddinge University Hospital.

Liver biopsy specimens and peripheral blood samples

Forty-three core needle biopsy specimens were obtained during the LTX, after revascularization of the new liver graft ($n = 9$), or within the 1st post-transplant year when the patient's liver function tests indicated graft dysfunction ($n = 34$; Table 1). The majority – 40 biopsy specimens – were obtained during the first 3 months after LTX. A modified Vim-Silverman needle (Tru-Cut disposable biopsy needle, outer diameter 2.0 mm; Travenol Laboratories, Deerfield Park, Ill., USA) was used for the perioperative biopsies. Post-LTX liver specimens were obtained with a Hepafix needle, 1.6 mm in diameter (B. Braun Melsungen, Melsungen, Germany). Between one and seven biopsy specimens were examined per patient. A minimum of 15 mm of the liver biopsy core was fixed in 4% formaldehyde and embedded in paraffin. Sections stained with hematoxylin-eosin were subjected to histopathological investigation ($n = 43$). Deparaffinized sections from these biopsies were used for EBV RNA in situ hybridization (EBER-ISH) and for immunostaining with ZEBRA, an EBV-lytic transactivator ($n = 41$; insufficient material in 2/43). A smaller part of the biopsy (2–5 mm in length) was fresh-frozen and kept at –70 °C for the retrospective nPCR examination for EBV DNA ($n = 43$). Thus, a total of 41 biopsy specimens were examined with EBER-ISH, ZEBRA, and nPCR, and the results were related to the histopathological diagnosis.

A serum sample was usually obtained on the same day (± 2 days) as the liver biopsy specimen and was also kept at specimen –70 °C for EBV-nPCR. The total number of available serum samples of good quality was 30. EBV IgG and IgM antibodies to the replicative antigens in P3RH1 cells (mainly the virus capsid antigens, VCA) and to EBV nuclear antigens (EBNA) were determined in serum by immunofluorescence, according to published methods [23].

Epstein-Barr virus genome analyses

The nPCR on both liver biopsy specimens and serum was performed using primers directed to a 147-bp-long fragment of the BAM HI 1 region encoding for EBNA 1 [3, 11, 13]. Cellular DNA from thawed liver biopsy specimens was extracted using the phenol/chloroform method, with minor modifications [10]. The EBV-nPCR was performed in duplicates of 10-µl aliquots of extracted DNA on one occasion. The examination was repeated if only one of the duplicates was positive. The EBV-nPCR on liver biopsy specimens was regarded as positive if two of four analyses turned out positive. Every seventh nPCR tube was a negative control.

In each nPCR examination, three dilutions of P2HR1-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. An inhibition control was performed using 1 µl of P3HR1-positive control per 10 µl of extracted DNA from liver biopsy specimens, giving a dilution of the positive control equal to the borderline control. If a sample with added positive control for the inhibition analysis did not show a positive EBV-nPCR, it was supposed to contain a blocking component. The biopsy specimen material was sufficient for the inhibition analysis in 38 cases.

EBV DNA was extracted from serum, as described previously [3, 11, 13]. The QIA amp blood kit (Quiagen, Hilden, Germany) was used according to 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. The EBV-nPCR was performed on duplicates of 10-µl aliquots of extracted DNA. The examination was repeated if only one of two was positive in order to rule out possible contamination. The EBV-nPCR on serum samples was considered positive when EBV DNA could be amplified in two of four sample sets.

The EBV-encoded small RNA-1 (EBER-1) was detected by EBER-ISH in formalin-fixed, paraffin-embedded liver biopsy specimens [41]. Briefly, the 5-µm-thick sections were deparaffinized, treated with 0.2 N HCl for 10 min at room temperature and with 5 µg of proteinase K (Sigma, St. Louis, Mo., USA) for 15 min at 37 °C. After dehydration, EBER-1 was probed with an anti-EBER-1 sequence labeled with FITC [10]. The hybridization occurred at 37 °C for 2 h. The FITC were detected with alkaline phosphatase-conjugated sheep anti-FITC (Boehringer, Mannheim, Germany) and visualized using BCIP/NBT (Dakopatts, Glostrup, Denmark). The double-labeling staining was performed after EBER-ISH procedures, using mouse monoclonal anti-keratin (clone MNF116, Dakopatts). The anti-keratin antibody was visualized with the streptoavidin-biotin complex method. Immunostaining for lytic infection (ZEBRA) was assessed with anti-BZLF1 antibody (BZ-1, Dakopatts). BamHI Z left frame 1 (BZLF1) protein is a transactivator for EBV lytic infection. After autoclaving and blocking, BZ-1 (1 : 20) was applied on the sections at 4 °C overnight. Then, BZ-1 was also detected by the streptoavidin-biotin complex method. For EBER-ISH, paraffin-embedded lymphoblastoid cell line (LCL) cells and a Hodgkin lesion, known to be EBV-positive, were used as positive controls. BJAB cells were used as negative controls. For BZLF1 detection, paraffin-embedded B95-8 cells, a EBV-producing cell line, were used as a positive control.

Histopathology of liver biopsy specimens

The histopathological evaluation of the liver biopsy specimens was made by one of the authors (F.P.R.), an experienced transplantation pathologist. The histological diagnosis of acute transplant rejection in each biopsy specimen was based on the Banff criteria [2]. According to these criteria, acute rejection was graded as 1, 2 or 3 (mild, moderate, or severe). In addition, cases of ischemic preservation injury, infarction, functional cholestasis, acute hepatitis, acute cholangitis of nonrejection genesis, large duct obstruction, chronic rejection, nonspecific changes, and normal findings were recorded.

Viral hepatitis, probably due to EBV [1], was considered when mixed infiltrates of mononuclear cells with large, atypical lymphocytes, immunoblasts, plasma cells, and monocytes were observed in the portal tracts and extending past the limiting plate into the lobules and sinusoids. Large, atypical lymphocytes are round cells with plentiful eosinophilic cytoplasm and a sharply delineated, oval, round, or indented nucleus with a well-defined chromatin pattern. Parenchymal engagement was typically less prominent than in portal tracts and consisted of individual hepatocyte necrosis with surrounding inflammatory infiltrates and focal canalicular cholestasis. The occurrence of acidophilic bodies was considered as supportive evidence of viral hepatitis.

Cytomegalovirus (CMV) infection of the liver graft was diagnosed when histopathological changes in viral hepatitis or cholangitis were verified using immunohistopathological detection of CMV antigens by immunostaining with monoclonal antibodies against CCH2 (Dakopatts) or against CH12 and CH16–20, kindly provided by Dr. L. Pereira (Viral and Rickettsial Disease Laboratory, Department of Health Services, San Francisco, Calif., USA) and/or CMV DNA detection using nPCR [4, 5].

The patients' files were studied to compare the results of the viral analyses and the histological findings with clinical data, without any knowledge of the results of EBV-specific examinations.

Statistics

Fisher's exact test was used. A P value below 0.05 was considered significant.

Results

Nested polymerase chain reaction in liver biopsy specimens

EBV DNA was detected with nPCR in 13 of 43 liver graft biopsy specimens (30 %) obtained from 9 of 18 patients (50 %). Seven EBV-nPCR-positive biopsy specimens were obtained in the first 3 post-LTX weeks and 12 specimens were obtained in the first 3 post-LTX months, i.e., 7/29 (24 %) and 12/40 (30 %) of the specimens obtained during the respective periods. None of the nine biopsy specimens obtained during LTX, shortly after revascularization, showed EBV DNA. Six patients

were EBV-nPCR-positive on one occasion, two patients were positive on two occasions, and one patient was positive on three occasions.

EBV DNA was found in three of eight biopsy specimens (38%) with histopathological features of acute cholangitis or hepatitis, in three of seven specimens (43%) with nonspecific histological changes, and in one of two specimens (50%) with changes due to ischemia, none of which was associated with preservation injury (Table 1). In two cases with nonspecific changes and in the two cases with signs of ischemia, slight portal inflammation with lymphocytes and plasma cells predominated. No EBV DNA was amplified from two specimens showing signs of large bile duct obstruction, although lymphocytic inflammatory infiltrates were found around bile ducts similar to the four cases mentioned above with nonspecific and ischemic changes in portal areas. In addition, one biopsy specimen without amplifiable EBV DNA had lymphocytic-immunoblastic infiltrates, reported to be characteristic of EBV disease in portal areas. Four of 12 biopsies (33%) with histopathology indicating acute rejection were EBV-nPCR-positive. The remaining two EBV DNA-positive biopsy specimens showed signs of chronic rejection.

Thus, EBV DNA was found in 6 of 15 specimens (40%) with inflammatory features and in 7 of 28 specimens (25%) without inflammation ($P = 0.32$, Fisher's exact test). When the biopsies obtained after revascularization during LTX ($n = 9$) were excluded, EBV DNA was found in 6 of 15 specimens (40%) with inflammatory features and in 7 of 19 specimens (37%) without inflammation ($P = 1.0$, Fisher's exact test).

Inhibition of added control EBV DNA occurred in 16/38 biopsy specimens (42%) with material available for the analysis. EBV-nPCR was negative in 12 (75%) and positive in 4 (25%) of the 16 specimens. However, EBER-ISH was positive in 5 of 12 nPCR-negative samples showing suspected nPCR blocking.

In situ hybridization and immunohistology in liver biopsy specimens

In situ hybridization (EBER-ISH) demonstrated EBV RNA in 14 of 41 available liver graft biopsy specimens (34%) obtained from 10 of 18 patients (56%). Seven specimens were obtained within 3 weeks and 12 specimens within 3 months post-LTX, i.e., 7/27 (26%) and 12/38 (32%) of the total number of specimens obtained during the respective periods (Table 1). One of the nine perioperatively obtained biopsies was EBER-ISH-positive. Six of 15 specimens with inflammation (40%) and 8 of 26 specimens without inflammation (31%) revealed EBV RNA ($P = 0.73$, Fisher's exact test). When the nine perioperative biopsies were excluded, 6 of 15 specimens with inflammation (40%) and 7 of 17 specimens without inflammation (41%) revealed EBV RNA ($P = 1.0$, Fisher's exact test). The positive controls were consistently positive and the negative controls were negative in EBER-ISH.

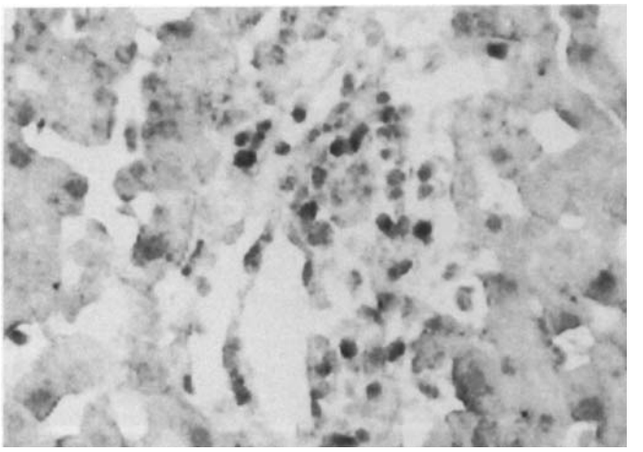

Fig. 1 Liver graft biopsy specimen demonstrating portal area. Note marked dark staining of nuclei of Epstein-Barr virus-infected mono-lymphocytic cells detected using in situ hybridization with EBER-1 (magnification × 250)

Immunostaining against ZEBRA, an EBV-encoded replication activator protein, was negative in all biopsy specimens. ZEBRA-positive cells were found scattered throughout the paraffin-embedded B 95-8 sections used as controls.

Comparison of nPCR and ISH findings with histopathology in liver biopsy specimens

There was a significant correlation between EBER-ISH and EBV-nPCR results since 9 of 41 specimens were concomitantly positive and 23 of 41 specimens negative using both methods ($P = 0.0033$, Fisher's exact test). Of the nine discordant samples, five were positive in ISH and four in nPCR. For the five samples positive in ISH, the PCR was probably blocked by inhibitor. Two EBV-nPCR-negative but EBER-ISH-positive biopsy specimens revealed signs of cholestasis due to suspected obstruction of a large bile duct. Immunohistological staining showed mononuclear inflammatory cells, passing the limiting plates of portal areas and in sinusoids, to be EBV RNA-positive (Fig. 1). In addition, EBV-nPCR negativity and EBER-ISH positivity occurred in one specimen with histological signs of acute cholangitis and in a further two specimens, obtained during the LTX and at the 1-year control, without inflammatory changes, respectively. EBV-nPCR positivity and EBER-ISH negativity were observed in three biopsies showing acute hepatitis, acute cholangitis, and nonspe-

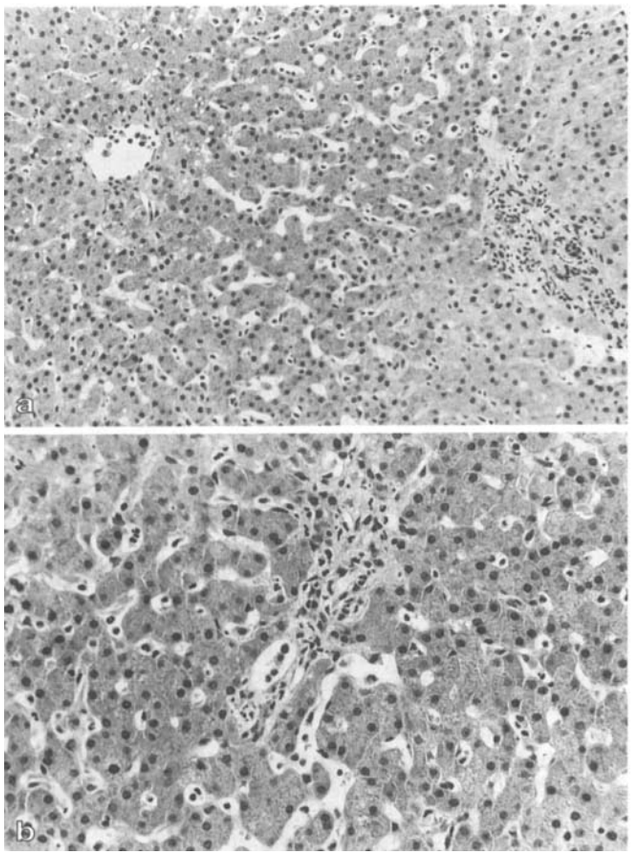

Fig. 2 **a** Low-power micrograph of liver graft biopsy specimen with slight hepatitis. The pathological changes are dominated by an increased number of lymphocytes in the sinusoids and slight inflammation in the portal tract. Centrilobularly, there is minimal vacuolization of hepatocytes as in fatty change (Hematoxylin and eosin × 80). **b** Medium-power micrograph of portal and periportal areas. There is slight portal inflammation with some lobular overflow but no clearcut, piecemeal necroses. Note also the slight sinusoidal inflammation with lymphocyte dominance (Hematoxylin and eosin × 156)

cific features with inflammation, respectively. In addition, one biopsy showing grade 3 acute rejection exhibited this discrepancy.

In two of eight specimens with histopathological signs of acute cholangitis or acute hepatitis, and in one of seven specimens with nonspecific features, the changes were suspected to be of viral genesis, due to hepatic inflammation and the occurrence of acidophilic bodies. The inflammatory cells infiltrating portal areas were lymphocyts, some of them blast-transformed or atypical, and plasma cells. Sinusoids were also infiltrated by histiocytic and monocytic cells (Fig. 2). Parenchymal changes were subtle, with single hepatocyte necrosis, often surrounded by some neutrophils, and signs of regeneration as illustrated by the occurrence of mitotic figures. EBV DNA was detected using nPCR in two specimens with changes of suspected viral genesis, i.e., the case of acute hepatitis and the case of nonspecific inflammatory features. The latter specimen belonged to the only liver recipient who was EBV-seronegative before transplantation, suggesting primary EBV infection. The third case could be explained by CMV, since the biopsy specimen was found to be positive for CMV using virus isolation, nPCR, and immunohistological (CCH2 and CH16–20) techniques. Thus, none of the three biopsies with histological signs indicating a viral infection were EBER-ISH-positive.

One of the patients with acute cholangitis, having concomittant EBV DNA both in the liver biopsy specimen and serum samples, and EBER-ISH positivity in the 1st post-LTX month developed EBV-associated monoclonal lymphoma in the lungs and abdomen 6 months after LTX. This patient has survived after reduced immunosuppressive treatment and chemotherapy.

Comparison of EBV nPCR in serum with EBV findings in liver biopsy specimens

EBV DNA was found in 8 of 30 available serum samples (27%) obtained from 16 patients simultaneously with the liver biopsy specimens. In half of the samples (4/8), EBV DNA was detected in serum simultaneously with the EBV-nPCR- and EBER-ISH-positive liver biopsy specimens. On four occasions, the serum sample obtained concomitantly with the EBV-nPCR-positive biopsy specimen was not available. However, in these four cases and in another four cases with EBV-nPCR- and EBER-ISH-positive biopsy specimens, a further serum sample obtained within 1 month contained EBV DNA. Two of the latter four patients had previous or ongoing ganciclovir treatment against CMV infection and CMV hepatitis, respectively.

The remaining EBV-nPCR-positive biopsy specimen in the total of 13 cases came from a pediatric patient without amplifiable EBV DNA in serum on days 0, 17, 28, and 102 post-LTX. The EBV-nPCR-positive but EBER-ISH-negative biopsy specimen was obtained on day 102. At that time, the patient was on ganciclovir treatment since day 13 post-LTX due to primary CMV syndrome, which later worsened to CMV hepatitis. The other three patients receiving ganciclovir treatment for CMV infection or disease were EBV-nPCR-negative in serum samples during treatment or within 14 days after treatment. The only EBER-ISH-positive biopsy combined with an EBV-nPCR-positive serum sample was obtained at the 1-year follow-up control. Interestingly, the patient had undergone a gastric resection because of an early gastric adenocarcinoma 5 months before LTX and had EBV DNA in pre-LTX serum. Immunohistology of the carcinoma specimen revealed no EBV DNA.

EBV findings in relation to antirejection treatment

The 13 EBV-nPCR-positive and 14 EBER-ISH-positive liver biopsy specimens came from nine liver recipients, i.e., 50% (9/18) of the study population. EBV DNA was observed in biopsy specimens and/or in parallel serum samples from five of nine patients (56%) receiving OKT3 treatment. In addition, three other patients given OKT3 treatment were later found to have EBV DNA in serum without any parallel liver biopsy specimen available for examination. Thus, the finding of EBV-nPCR positivity tended to occur more often in liver biopsy specimens and serum samples of OKT3-treated patients (8 of 9; 89%) than in those of patients in whom high-dose steroids had been successfully used to treat rejection (4 of 9; 44%; $P = 0.13$, Fisher's exact test).

Discussion

EBV DNA and EBV RNA were detected in 30% and 34% of liver graft biopsy specimens, respectively, obtained from 50% of the patients within the 1st post-LTX year. Most of the EBV-positive biopsy specimens – 30% using nPCR and 32% using ISH – were obtained during the first 3 months postoperatively. There was a significant association between EBV-nPCR- and EBER-ISH-positive biopsy specimens ($P = 0.003$). None of the biopsy specimens revealed lytically EBV-infected cells using ZEBRA immunostaining. All but one patient had an EBV DNA-positive serum sample, obtained within 1 month after taking the liver biopsy specimens. Histopathological features suggestive of EBV hepatitis were found in three biopsy specimens with positive EBV-nPCR but negative ISH staining in two cases. The third case was associated with CMV.

It is assumed that all seropositive individuals harbor small numbers of EBV latently infected lymphocytes in their peripheral blood and tissue. The high sensitivity of PCR allows viral DNA from such cells to be amplified, in addition to DNA from cells with EBV replication. A positive PCR signal for EBV in a liver biopsy does not establish whether the infection is in the liver cells – and therefore of potential pathogenic importance – or whether the virus is merely in infiltrating inflammatory cells [15]. However, in immunosuppressed transplant recipients, the mere presence of EBV may indicate a risk of EBV-associated complications. Since most of the liver recipients and donors in this study were verified or assumed to be EBV-seropositive, the risk of EBV reactivation or reinfection was obvious. In addition, there is always a risk of EBV transmission by perioperative blood transfusions due to the high prevalence of EBV in the population. Therefore, we have developed our nPCR to be highly sensitive [11], as shown in the rate of PCR-positive biopsy specimens. The sensitivity is higher than that in a study by Alshak et al. [1]. Alshak et al. found EBV DNA in only 3 of 61 samples. These were histopathologically highly suspicious of hepatitis caused by EBV.

EBERs are nontranslated, small viral RNAs produced in large amounts in all forms of EBV latency [18]. However, EBER has been detected at lower rates than EBV DNA, detected by nPCR, in tissue sections [34, 36]. The sensitivity of ISH depends on the extent of probe penetration, and on the efficiency (length of the probe) and specificity of the probe. This may explain the paucity of nPCR-positive and EBER-ISH-negative results of liver biopsy analyses in this study. In addition, EBV replication had been shown by the absence of a detectable latent phase and EBER in oral hairy leukoplakia [30, 37]. A similar pattern may occur in immunosuppressed transplant recipients, as indicated by the positive nPCR and negative EBER-ISH in patients with histopathological changes compatible with EBV infection in this study. Such observations have also been made by others [34]. However, stainings for ZEBRA were negative for our two patients with this finding. Since staining of formalin-fixed and deparaffinized tissue may sometimes have a low sensitivity, ruling out or confirming lytic infection in liver disease needs further investigation.

The length of the liver biopsy specimen used for EBV DNA amplification was 2–5 mm. This should be compared to the 15–20 mm available for histopathological, immunohistological, and EBER-ISH analyses. Thus, a very small specimen might explain the false-negative nPCR with EBER-ISH-positive specimens. The main reason, however, for the discrepancy seems to be substances blocking the nPCR in some DNA preparations. Bile salts and bilirubin are substances that may inhibit DNA amplification [21]. Five of 12 biopsy specimens inhibiting the PCR reaction due to a suspected blocking factor were EBER-ISH-positive. If these five samples were excluded from the comparison between ISH and nPCR, the correlation between nPCR and EBER-ISH would be even better.

Half of the EBV-infected biopsy specimens were obtained as early as the first 3 weeks post-LTX. This reflects the very high doses of immunosuppressants needed for antirejection treatment in 80%–90% of liver recipients during the 1st month after LTX. All immunosuppressive drugs used in the present study – CyA, tacrolimus, and anti-CD3 cell monoclonal antibodies – block T-lymphocyte inhibition of EBV-infected B lymphocytes and allow B-cell proliferation [8, 9, 12, 28]. In our study, patients with additional anti-CD3 cell antibody (OKT3) treatment tended to develop an EBV infection more often than in previous studies [3, 25]. In addition to EBV hepatitis, the defective regulation of EBV may allow the development of EBV-related lymphoproliferative disorders [14, 16, 29, 30, 35, 42]. One patient in the present study developed EBV-associated

lymphoma with manifestations in the lungs and abdomen.

Histological evaluation of a liver graft biopsy is important for distinguishing rejection from toxic or infectious causes of pathological liver function tests in peripheral blood since the type of treatment varies with the cause. The histological differential diagnosis between allograft rejection and EBV infection may be difficult, especially in the early postoperative period. A diagnosis of acute rejection is based on the classical triad of histological changes: portal inflammation, bile duct destruction, and venous endothelitis [2]. The typical inflammatory infiltrate in acute rejection is of lymphocytes, some of which may be blast-transformed, mixed with monocytes/macrophages and often a considerable number of eosinophils. The lymphocytic-immunoblastic infiltrate described as characteristic of EBV infection in biopsy specimens after LTX [1] is by no means diagnostic unless the so-called atypical large lymphocytes are plentiful. In most cases, the bile duct involvement in EBV hepatitis is mild, despite an intense portal inflammation, although intraepithelial infiltration of lymphocytes is sometimes observed, often along with canalicular cholestasis [24, 39]. In acute rejection, however, bile duct involvement, which frequently is destructive, is characteristic. Perhaps the most discriminating feature between rejection and EBV hepatitis is venous endothelitis, a nearly obligatory finding in acute rejection. Although sinusoidal inflammation occurs in EBV hepatitis after LTX, venous endothelitis has, as far as we know, not been reported in EBV hepatitis. From the discussion above, it is obvious that a concomitant EBV hepatitis cannot be excluded in the case of acute rejection. On the other hand, a typical EBV hepatitis may, in most cases, be differentiated from a episode of acute rejection.

One particular problem is that the viral infection often start during treatment of an acute rejection episode, when the patient is under adjunct immunosuppressive therapy and the immune reaction is hampered. This may be an alternative explanation to the 30%–40% of EBV-nPCR-positive and/or EBER-ISH-positive biopsy specimens, where features of acute or chronic liver graft rejection or nonspecific inflammation were observed following rejection without characteristic lymphocytic-immunoblastic inflammatory infiltrates of EBV hepatitis. In a similar number of cases (36%–43%), infiltrates of monocytes, lymphocytes, immunoblasts, and plasma cells, especially in the portal tracts and extending past the limiting plate into the lobules and sinusoids, were present in combination with positive EBV-nPCR and/or EBER-ISH. Thus, our findings are only to some extent in line with the results of Alshak et al., who reported the latter type of histopathological features to be a reliable marker of EBV hepatitis [1]. Such a pattern of inflammation should, however, arouse suspicion of possible EBV infection. Changes compatible with viral infection, such as enlarged cells and acidophilic bodies, were only seldom observed; they are unspecific and correlated not only with EBV but also with CMV in this study. In addition, sinusoidal lymphocytic-histiocytic infiltrates are reported in other types of viral hepatitis, such as hepatitis C virus [20, 27].

The concomitant occurrence of EBV DNA in serum, found in the vast majority of patients, strengthens the suspicion of EBV-associated liver infection [3, 34]. Confirmatory methods to demonstrate EBV etiology of an inflammatory liver disease, such as ISH or PCR, must be used. However, a positive PCR or ISH does not necessarily point to a symptomatic liver infection with EBV. Virological and histopathological findings evaluated together are the only reliable basis for a firm diagnosis of EBV hepatitis. The combination of histopathological features of lymphocytic-immunoblastic infiltrates, especially in portal areas with parenchymal changes, suggestive of hepatitis, and findings of EBV genome, using nPCR and ISH in colocalized inflammatory cells, allowed us to diagnose EBV hepatitis in 2 of 18 liver recipients.

In conclusion, EBV-nPCR, if possible combined with EBER-ISH, is recommended in examinations of biopsy specimens of transplanted livers, especially when EBV DNA is found in the serum. Histopathological changes in EBV hepatitis may be nonspecific and complicated by parallel or remaining signs of acute rejection or other conditions. A quantitative EBV DNA method may be valuable for monitoring patients at risk of EBV disease, such as those given antilymphocyte treatment.

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