JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1999, p. 1113–1116 Vol. 37, No. 4
0095-1137/99/$04.00+0

Detection of Epstein-Barr Virus DNA in Sera from Transplant Recipients with Lymphoproliferative Disorders

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Received 9 October 1998/Returned for modification 7 December 1998/Accepted 13 January 1999

Early diagnosis of Epstein-Barr Virus (EBV)-associated posttransplant lymphoproliferative disease (PTLD) is important because many patients respond to reduction in immunosuppression, especially if PTLD is detected at an early stage. Previous studies have found elevated EBV DNA levels in blood from patients with PTLD, but these assays required isolation of cellular blood fractions and quantitation. We evaluated the presence of cell-free EBV DNA in serum from solid-organ transplant recipients as a marker for PTLD. Five of 6 transplant recipients with histopathologically documented PTLD had EBV DNA detected in serum at the time of diagnosis (sensitivity = 83%), compared with 0 of 16 matched transplant recipients without PTLD (specificity = 100%) ($P < 0.001$ [Fisher's exact test]). Furthermore, EBV DNA was detected in serum 8 and 52 months prior to the diagnosis of PTLD in two of three patients for whom stored sera were analyzed. Detection of EBV DNA in serum appears to be a useful marker for the early detection of PTLD in solid-organ transplant recipients. Further studies to define the role of such assays in evaluating solid-organ transplant patients at risk for PTLD are warranted.

Posttransplant lymphoproliferative disease (PTLD) is an uncommon but important cause of morbidity and mortality in organ transplant recipients; it results from uncontrolled Epstein-Barr virus (EBV)-induced proliferation of B cells in the setting of immunosuppression (7, 11, 18, 28). Early detection is critical, since reduction in immunosuppression may result in regression of PTLD (5, 26). Given the close association of EBV and PTLD, both invasive and noninvasive strategies have been devised to monitor patients for EBV after transplantation (10, 13, 15, 17, 19, 20, 22–25, 29). Previous studies have reported high levels of EBV DNA in the blood of patients with PTLD as determined by assays designed to detect EBV DNA in peripheral blood lymphocytes (PBL) (9, 10, 15, 17, 22–25). The assays used in these studies required separation of lymphocytes and/or mononuclear cell fractions from whole blood. But, since latent EBV DNA is present in EBV-seropositive persons, the demonstration of quantitative differences between EBV DNA levels in patients with and without PTLD was required when this sample source was utilized.

We have sought to address some of these issues by evaluating whether we could detect EBV DNA directly from serum and by analyzing sequentially collected sera from transplant recipients who subsequently did or did not develop PTLD. We hypothesized that detection of EBV DNA in acellular specimens (i.e., serum) would occur only for patients with very high systemic EBV genome loads and thus might better differentiate patients with PTLD from transplant recipients with low levels of EBV reactivation as a result of immunosuppression.

MATERIALS AND METHODS

Patients and specimens. All recipients of abdominal (kidney, pancreas, liver) transplants with histopathologically confirmed PTLD were identified prospectively during the study period (1 January 1996 through 30 June 1998) at the University of Washington Medical Center (Seattle). PTLD was diagnosed according to previously published clinical and histologic criteria (16, 19). All biopsy specimens were analyzed by routine histochemical stains, flow cytometry (cell surface markers and determination of clonality), and both immunocytochemistry and in situ hybridization for EBV-specific products as previously described (4, 16, 19, 27).

Six patients were diagnosed with PTLD during the study period, and in each case, a sample of serum or plasma was obtained within 1 week of the diagnosis of PTLD and stored at −70°C until analyzed. Control patients included 16 transplant recipients who were matched with regard to the organ transplanted, pretransplant EBV serostatus, and immunosuppressive regimen used. Each control patient had a minimum of 1 year of follow-up (or until death) after the date of the last serum sample collection to determine whether PTLD had developed.

A total of 23 serum samples from the 6 case patients with PTLD and 87 serum samples from the 16 matched, control patients were analyzed. The collection dates of the 23 samples from the case patients ranged from 0 to 79 months posttransplant (median of 5.5 months posttransplant), while those from the control patients ranged from 0 to 72 months posttransplant (median of 4 months posttransplant). Five of the 6 case patients (83%) and 15 of the 16 control patients (94%) were EBV seropositive at the time of transplant.

EBV PCR. All PCR analysis was performed by persons who were blinded to the clinical status of the patient whose sera were being analyzed (i.e., PTLD case versus control). The EBV PCR assay was performed as previously described (12). DNA was extracted from 200 μl of serum by using the QIAamp blood kit (Qiagen) and was eluted with 100 μl of 10 mM Tris (pH 8.0). Ten microliters of DNA was used for each PCR. Primers EBER1-A and EBER1-B (GTT TTG CTA GGG AGG AGA CG and GAC CGA AGA CGG CAG AAA GC, respectively) were used to amplify a 118-bp region of EBER1 (1). Each 100-μl PCR mixture contained 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.4); 2 U of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, Calif.); 200 μM (each) dGP, dATP, and dCTP; 400 μM dUTP (Pharmacia); a 0.83 μM concentration of each primer; 10 μl of glycerol; and 10 μl of purified DNA template. PCR conditions were as follows: 96°C for 2 min; 35 cycles of 94°C (30 s), 54°C (30 s), and 72°C (30 s); and 72°C for 5 min. The EBER1-A–EBER1-B PCR product was detected by liquid hybridization with ^{32}P-labeled probe, EBER1-P (GAC ACC GTC CTC ACC ACC CG) (12). Seven microliters of PCR product and the probe (106 cpm) were heated in 25 μl of a solution containing 1.2 M NsCl, 100 μM each deoxynucleoside triphosphate, and 44% formamide at 97°C for 5 min. Ten-microliter samples of cooled hybridization products were analyzed on 6% acrylamide gels, dried, and then autoradiographed. To ensure that negative results were not due to nonspecific inhibition of PCR, each PCR product was spiked with 50 copies of EBER-fly2 DNA. EBER-fly2 is identical to the EBER1-A–EBER1-B PCR product except that the EBER1-P probe sequence is replaced by 21 bp of *Drosophila* DNA (6). Each EBER1-A–EBER1-B PCR product was also hybridized with ^{32}P-labeled fly probe, electrophoresed, and then autoradiographed. All the negative EBER1-A–EBER1-B PCR results required detection

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TABLE 1. Clinical characteristics of patients with PTLD[a]

PTLD patient	Age (yr)/sex	Transplant type	Pre-Txp EBV serostatus	PTLD diagnosis time (mo)[b]	EBV PCR result at PTLD diagnosis	PTLD site(s) or symptom
1	49/M	Liver	+	7.5	+	Lung, bone
2	45/M	Liver	+	13	+	Spine
3	43/F	Kidney/pancreas	+	36	+	Lung
4	41/M	Kidney	+	70	+	Abdominal LN
5	30/M	Kidney	−	33	−	Skin nodule
6	44/M	Liver	+	79	+	Diffuse LN

[a] Abbreviations: M, male; F, female; Txp, transplant; LN, lymphadenopathy.
[b] Number of months after transplant until PTLD was diagnosed.

of EBER-fly2 DNA. To monitor for false positives, specimens were processed in parallel with aliquots of phosphate-buffered saline. PCR mixtures without DNA were also included in each PCR run. To ensure reproducibility of the PCR result, each specimen was run in duplicate and the assay was repeated for each specimen.

Statistical analysis. Fisher's exact test on a two-dimensional contingency table was used to compare the EBV PCR assay results for patients with PTLD and control patients.

RESULTS

Six patients were diagnosed with PTLD during the study period; the clinical characteristics of these patients are shown in Table 1. Five of the six patients were EBV seropositive prior to transplant. The time of diagnosis of PTLD ranged from 7.5 months to 79 months after transplant. All biopsy specimens met criteria for PTLD (16, 19), and all specimens were determined to contain EBV-related products by immunocytochemistry and/or in situ PCR.

EBV DNA was detected in the sera of 5 of 6 patients at the time of diagnosis of PTLD. In contrast, EBV DNA was not detected in the serum of any of the 16 matched control patients ($P < 0.001$ [Fisher's exact test]) (Table 2). The single patient for whom EBV DNA was not detected in serum at the time of diagnosis of PTLD presented with a solitary skin nodule and no evidence of disease elsewhere (patient 5) (Table 1).

A total of 87 serum samples from 16 control patients were tested, and all were negative for EBV DNA by our assay (Table 2). Stored sera from three of the patients with PTLD were available for analysis, and the results are shown in Table 3. For two of the three patients, EBV DNA was detectable in serum as early as 8 and 52 months prior to the diagnosis of PTLD (patients 1 and 3, respectively). For patient 2, only two stored serum samples were available, collected 13 and 19 months prior to the diagnosis of PTLD, and both were negative for EBV DNA in our assay.

DISCUSSION

Noninvasive monitoring could theoretically be a useful method for evaluating transplant patients at risk for developing PTLD and might provide a basis for the use of preemptive strategies, such as reduction in immunosuppression or antiviral therapy, to reduce the incidence and severity of PTLD after transplantation. Several studies have assessed EBV DNA levels in transplant recipients with PTLD and have demonstrated elevated levels in PBL from patients with PTLD compared to controls (10, 15, 17, 19, 22–25, 29). All of these studies used assays that required the separation of cellular fractions from blood (i.e., either peripheral blood mononuclear cells or PBL) and required quantitation. In the present study, a technically simpler method of EBV DNA amplification directly from serum was used to analyze samples from solid-organ transplant recipients with and without PTLD.

We evaluated the sensitivity of PCR in serum to provide a screening method for PTLD, thereby obviating the need for both separation of cellular fractions from whole blood and quantitation of levels if EBV DNA was detected. We hypothesized that detection of EBV DNA in serum (as opposed to PBL) would occur only for patients with relatively high systemic EBV loads and thus would better differentiate transplant recipients with and without PTLD than assays that measure EBV DNA levels in PBL. Barkholt et al. published a study in which EBV DNA was measured in the sera of a cohort of liver transplant recipients at risk for developing PTLD (3). A nested-PCR assay was used, and 58% of all patients had detectable EBV DNA levels at some point within the first 3 months posttransplant. The assay's sensitivity was such that 33% of patients had EBV DNA detectable in serum even before liver transplantation. Although EBV DNA was detected in the serum of all three patients who subsequently developed PTLD, the high frequency of EBV detection in patients without PTLD limited the assay's specificity and clinical utility for predicting subsequent development of PTLD. In contrast, we used a nonnested PCR and found that 0 of 87 sera from 16 control patients had detectable EBV DNA at various times after transplant (specificity = 100%). Conversely, 5 of 6 prospectively identified patients with PTLD had EBV DNA detected in serum at the time of diagnosis (sensitivity = 83%). The only patient who was negative in the assay at the time of PTLD diagnosis had a single subcutaneous skin nodule (without any evidence of PTLD elsewhere by clinical exam or total body imaging) as the sole manifestation of PTLD. In the only other study of EBV DNA detection in acellular specimens, Fontan and colleagues documented EBV DNA in serum of patients with PTLD, but few details about the timing of the collection of the positive serum samples relative to the time of diagnosis or clinical characteristics of patients were provided (8).

If our findings are validated in larger studies, detection of serum EBV DNA could prove to be useful for evaluating solid-organ transplant patients with clinical syndromes compatible with PTLD. For example, two of the patients in the present study presented with pulmonary nodules and fever

TABLE 2. Serum EBV PCR results for transplant recipients who developed PTLD and those who did not develop PTLD

EBV PCR result	No. of patients with result[a]		No. of serum samples with result	
	PTLD ($n = 6$)	Control ($n = 16$)	PTLD ($n = 23$)	Control ($n = 87$)
Positive	5	0	20	0
Negative	1	16	3	87

[a] $P < 0.001$ (Fisher's exact test) for PTLD patients versus controls.

TABLE 3. Retrospective analysis of stored sera from patients with PTLD for EBV DNA

Patient	Date (mo/day/yr) of PTLD diagnosis	Serum sample collection date (mo/day/yr)	Presence of EBV DNA in serum
1	2/13/97	6/22/96	+
		7/18/96	+
		11/10/96	+
		11/17/96	+
		11/20/96	+
		11/25/96	+
		11/27/96	+
		12/3/96	+
		12/10/96	+
		12/17/96	+
		12/24/96	+
		1/7/97	+
		2/8/97	+
2	5/30/97	11/9/95	−
		4/15/96	−
		6/1/97	+
3	11/9/97	7/16/93	+
		11/8/94	+
		2/27/95	+
		11/6/97	+

(patients 1 and 3). A variety of infectious and noninfectious etiologies were considered, and detection of EBV DNA in sera from these patients led us to temporarily reduce immunosuppression (as empiric treatment for possible PTLD) until a definitive invasive procedure (i.e., open lung biopsy) could be performed and the diagnosis of suspected PTLD could be confirmed. In both cases, the results of the serum EBV PCR were available before histopathologic evaluation of the lung biopsy specimens. Thus, such an assay could potentially serve as a useful adjunct to definitive histopathologic evaluation for the early diagnosis of PTLD.

In addition to potentially serving as an adjunct to the early diagnosis of PTLD, detection of EBV DNA in serum might be used to identify patients at risk for developing PTLD. In the present study, we were able to detect EBV DNA persistently in the sera of two of three patients who subsequently developed PTLD, thereby supporting a role for closely monitoring patients with EBV DNA detectable in serum. If our results are validated in larger studies, routine monitoring for EBV DNA in serum may provide a marker by which preemptive strategies are implemented (e.g., antiviral therapy or reduction of immunosuppression when EBV DNA is detected in serum). Only randomized prospective trials will clarify the effectiveness of such a strategy.

Several limitations of the present study should be noted. The numbers of patients were relatively small, and larger studies validating our results are necessary. Further, although the sera collected at the time of PTLD diagnosis were from prospectively identified patients, the other samples from the same patients were not necessarily collected at identical times for all patients. Moreover, some cases of PTLD develop after primary EBV infection, and acute EBV infection may be associated with serum viremia and thus not necessarily predict subsequent development of PTLD. This is an important consideration in the pediatric transplant population, where a significant proportion of patients are at risk for acquiring primary EBV infection.

In summary, we have used PCR to amplify EBV DNA directly from sera of patients with PTLD and have evaluated the assay by using a group of transplant recipients with and without PTLD. The ease of performance, sensitivity, and specificity of the assay for the early detection of PTLD appear to be promising, and further studies using this assay for monitoring patients for PTLD after solid-organ transplantation are warranted.

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