Direct Correlation Between the Load of Epstein-Barr Virus-Infected Lymphocytes in the Peripheral Blood of Pediatric Transplant Patients and Risk of Lymphoproliferative Disease

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The Epstein-Barr virus (EBV) is known to cause posttransplant lymphoproliferative disease (PTLD) in immunosuppressed transplant patients. The results of this pilot study showed that all EBV+ patients pretransplant experienced primary EBV infection within the first 3 months after transplant surgery. Virtually all of these patients had a higher burden of EBV-infected cells in their peripheral blood (PB) after infection by EBV than did the EBV− pretransplant group when tested at the same intervals posttransplant. Salivary EBV titers also increased in most patients, but the difference between the two groups was statistically significant only at 12 months, whereupon EBV+ patients showed higher titers compared with EBV− (α < 0.053). Also, polymerase chain reaction amplification followed by Southern blotting was performed to detect EBV sequences in PB mononuclear cells.

The Epstein-Barr virus (EBV) is a lymphotropic herpesvirus that infects and immortalizes human B lymphocytes. These cells are thought to preferentially maintain the viral genome in a nonreplicating latent form. Persistent lytic replication of EBV is supported by oropharyngeal epithelial cells. Productive infection in the oropharynx allows the virus to be transmitted horizontally via salivary exchange. Whereas childhood infection is usually asymptomatic, primary EBV infection later in life may give rise to the signs and symptoms characteristic of heterophile-positive infectious mononucleosis. Whereas immunocompetent individuals can limit proliferation of EBV-infected cells, those with congenital or acquired immunodeficiency are highly susceptible to EBV-associated lymphoproliferations. The majority of these B-cell lymphoproliferations were shown to harbor EBV DNA and/or EBV-specific antigens. Based on their histopathology, these tumors have been classified into two main groups: polymorphic diffuse B-cell hyperplasia and polymorphic B-cell lymphoma. Transplant patients are particularly at risk for developing posttransplant lymphoproliferative disease (PTLD) because of the heavy immunosuppressive regimens to which they must adhere to prevent graft rejection. Immunosuppressive drugs act by suppressing T-cell activity, thereby interfering with immune surveillance and increasing the risk of EBV-infected B-cell proliferation. A recent study showed that patients with PTLD showed a higher rate of primary or reactivated EBV infection compared with the general transplant population (93% for the former versus 30% for the latter). It has also been shown that patients undergoing primary EBV infection after transplantation were at greatest risk for development of PTLD: 43% of patients with tumors had primary EBV infection compared with 8% of patients without tumors.

The objective of this study was to determine whether the EBV load in blood and/or saliva might serve as a marker of immunosuppression in transplant patients. Using this information, we then sought to evaluate whether a correlation existed between risk of PTLD and level of immunosuppression, as determined by EBV burden in blood and/or saliva.

This technique allowed confirmation of the blood culture results and constituted a faster alternative compared with the culture assay. The highest increase in the number of EBV-infected lymphocytes at 3 months posttransplant obtained from PB was seen in a patient who developed fatal PTLD and in another with protracted infectious mononucleosis. Thus, the number of EBV-infected cells in PB was found to correlate positively with risk of development of PTLD at 3 months posttransplant in our group of pediatric transplant patients. This study showed that quantitative lymphocyte culture of PB was an accurate index of immunosuppression and a reliable method for assessing the risk of PTLD development.

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MATERIALS AND METHODS

Patient Selection

Sainte-Justine Hospital (Montreal, Quebec, Canada) pediatric transplant patients were selected for this study. Saliva and heparinized blood were collected before and at 3, 6, and 12 months posttransplant from three heart, five kidney and eight liver pediatric transplant patients. Thus, a total of 64 blood and 48 saliva samples were anticipated for processing, including determination of EBV-antibody titers to the EB nuclear antigens (EBNA), the viral capsid antigens (VCA) and the early antigens (EA), quantification of EBV burden in blood and saliva by culture, and semiquantitative analysis of EBV burden in peripheral blood (PB) after amplification by polymerase chain reaction (PCR). However, 54 blood and 48 saliva samples were actually received. Heparinized blood was also collected from the organ donors for EBV serology and culture.

Leukocyte Cultures

The number of EBV-infected cells in the PB was determined by Rocchi’s culture method. Briefly, serial 10-fold dilutions of the patients’ PB mononuclear cells (from 5 × 10⁶ cells/well to 5 × 10³ cells/well) were cultured in round-bottom 96-well microplates with 6 × 10⁵ cord blood mononuclear cells/well, using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), antibiotics and 0.5 μg/mL cyclosporine A. Each dilution was cultured in six repli-

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cate wells. The transformation titer was determined by calculating the number of wells transformed per total number of patient-derived cells seeded at the highest dilution where transformation was still visible. All titers were reported as number of cells transformed per 10^6 total patient-derived mononuclear cells.

**Saliva Cultures**

Throat washes were obtained and titered for EBV by transformation of cord blood lymphocytes. Approximately 1 mL saliva was obtained by suction and oropharyngeal epithelial cells were obtained by scraping the inside of the cheek, directly over the parotid duct opening. The cell pellet, recovered after low-speed centrifugation, was divided in 2 equal aliquots. One of these was stored frozen for subsequent PCR analysis (see below). The other was subjected to three freeze-thaw cycles to free cell-bound virus. Cell debris was eliminated by low-speed centrifugation and the supernatant fluid was filtered through a 0.45-μm syringe filter unit. A modification of the lymphocyte transformation assay described by Chang et al. was used to obtain viral titers in saliva. Briefly, cord blood mononuclear cells were plated to 250,000 cells per well in 96-well round-bottom microplates, using RPMI-1640 medium containing 10% FBS and antibiotics. Six replicate wells were inoculated with 10 μL of undiluted saliva or twofold serial dilutions thereof (twofold, fourfold, and eightfold dilutions of original sample). The viral titer was determined using the following formula:

\[
\frac{\Sigma (\text{no. positive wells} \times \text{dilution})}{\Sigma (\text{total wells} \times \text{dilution})} \times 100
\]

**Indirect Immunofluorescence Microscopy**

Indirect immunofluorescence microscopy with the PE2 MoAb for detection of EBNA-2 was performed on cultured lymphocytes as previously described. This was done to confirm B-cell transformation by EBV.

**Treatment of Samples for PCR Amplification**

PB mononuclear cells were pelleted, washed in phosphate-buffered saline and resuspended in deionized H2O to the order of 10^6 cells/mL. The suspension was boiled for 10 minutes in preparation for PCR amplification (see below). Similarly, 20 μL of saliva cell pellet was boiled in 0.5 mL H2O before PCR amplification. The primer pair and oligonucleotide probe were generated from the BMLFL1 region of the EBV genome, as described by Pedneault et al. The predicted sequence length of the amplified product was 304 bp. PCR amplification of each sample was based on the method described by Saiki et al. Briefly, the reaction mixture (100 μL) contained a final concentration of 20 mmol/L TRIS-HCl (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L MgCl2, 0.1% Triton X-100, 0.01% (wt/vol) gelatine, 1.0 μmol/L each primer and 200 μmol/L each deoxyribonucleotide triphosphate: deoxyadenosine triphosphate (dATP), deoxyctydine triphosphate, deoxycytosine triphosphate, and deoxythymidine triphosphate. The mixture was overlaid with 75 μL mineral oil. Twenty microliters of the boiled cell suspensions was added through the oil. The reaction mixture was then heated to 95°C for 10 minutes and 4 μL (containing 2.5 U) Taq DNA polymerase (Pharmacia Biotech Inc, Baie d’Urfé, Quebec, Canada) was added immediately to the individual reaction tubes. In our laboratory, optimal conditions for amplification using the BamHI M primers consisted of a 30-cycle run, with each cycle programmed for 1 minute at 94°C (“hot start”) to denature the DNA, 1.5 minutes at 55°C for primer annealing and 1.5 minutes at 72°C for primer extension. At the end of the last cycle, the samples were incubated at 72°C for an additional 10 minutes to ensure completion of the extension step.

One positive and several negative controls were included in each run. Amplification of a segment of the HLA DQα gene sequence was performed using published primer sequences to confirm the presence of cellular DNA in each sample.

Southern transfer was performed to increase the sensitivity of the reaction and to authenticate the PCR-amplified product. A total of 10 μL of the PCR reaction product was electrophoresed on a 1.8% agarose gel. The gel was then treated with alkaline denaturing solution, neutralized, and transferred onto a GeneScreen Plus nylon filter membrane (Du Pont Canada Inc, Mississauga, Ontario, Canada), as per standard protocol. The membrane was then baked at 80°C for 2 hours and prehybridized for 2 hours in a solution containing 6× SSC (0.9 mol/L NaCl + 90 mmol/L Na citrate), 0.2% sodium dodecylsulfate (SDS), 5× Denhardt’s and 0.1 mg/mL denatured salmon sperm DNA. Hybridization was performed with end-labeled probe obtained using phage T4 polynucleotide kinase (Clontech Laboratories Inc, distributed by BIOCAN Scientific, Mississauga, Ontario, Canada) and [γ-32P]-dATP (Du Pont Canada/New England Nuclear, Mississauga, Ontario, Canada) to the order of 10^6 cpm/2 mL for an incubation period of 4 hours at 60°C. The membrane was then washed 4 times for 15 minutes each wash, as follows: 6× SSC + 0.1% SDS at 60°C, 3× SSC + 0.5% SDS at 60°C, 6× SSC + 0.1% SDS at 60°C, and 0.2× SSC at room temperature. The blots were exposed to XAR film (Picker International Canada Inc, St Laurent, Quebec) for 4 hours or overnight at ~86°C.

**EBV Serology**

EBV infection in our group of patients was monitored by serology, using an enzyme-linked immunosorbent assay kit (Gull Laboratories, distributed by BIOCAN Scientific) for measuring IgM to the EBV VCA and by indirect immunofluorescence microscopy on B95-8 cells for VCA IgG. Indirect immunofluorescence microscopy on Raji cells induced with 5-bromo-2’ deoxyuridine was used for detection of antibodies to EA. The EBNAs were detected by anticomplement immunofluorescence microscopy on uninduced Raji cells. A patient was considered EBV+ when all serologies, blood and saliva cultures, as well as PCR analyses were clearly negative. Primary EBV infection was defined by an unambiguous VCA-IgM* serology, a positive Paul-Bunnell-Davidsohn test and/or a positive leukocyte/saliva culture.

**Statistical Analysis**

The results obtained using PB were analyzed after performing a logarithmic transformation (log_{10}(x + 1)) of the original data to correct for the highly skewed distribution.

The statistical analysis was done in two stages. The first consisted of a set of evaluations performed separately on the data at times 3, 6, and 12 months, with an analysis of variance between groups using the SAS Statistical Package (SAS Institute Inc, Cary, NC). The second stage of analysis was performed to evaluate the variance for repeated measurements at 3, 6, and 12 months. The initial data at time 0 was ignored because no variance existed between the values. At this stage only those patients with a full set of measurements were selected, thereby excluding 4 subjects (patients 4, 5, 7, and 8) with incomplete data. All results were presented in arithmetic units after retransformation of the data to antilog_{10}(x − 1).

The data obtained for EBV titers in saliva was analyzed, with respect to group and time, using the Wilcoxon-Mann-Whitney formula, which allowed analysis of data that was not normally distributed. Time 0 was not considered because all but one sample yielded transformation titers above the baseline. Analysis of variance was considered significant at values of p < 0.05.
RESULTS

Case Histories of the Liver Transplant Patients Who Developed PTLD

Patient no. 4. This infant girl, born on November 23, 1990 with congenital biliary atresia, had two successive liver transplantations, performed on September 8 and September 11, 1991 with organs donated by EBV and CMV seropositive donors. She died on December 13, 1991 with culture-proven pulmonary CMV infections that did not respond to ganciclovir, and with lymphoproliferative disease (Ann Arbor grade IV/IV) in the transplanted liver. However, the intestinal tract was also affected, with multiple bleeding lymphomatous tumors, as were the cervical, mediastinal, and mesenteric lymph nodes. The identification of surface Ig by immunoperoxidase labeling and Ig gene rearrangements confirmed the oligoclonality of the tumors. A liver biopsy performed 1 week before death was positive for EBV by PCR analysis. Immunosuppressive therapy included immuran, cyclosporine, prednisone, and OKT3 (see Table 3).

Patient no. 6. This adolescent girl with type I glycogenosis underwent liver transplantation on October 27, 1991 at the age of 16 years and was placed on cyclosporine, immuran, and solumedrol/prednisione. OKT3 was added to the above from November 5 to November 15, 1991 because of signs of rejection, as judged by liver biopsy and analysis of liver transaminases. She was then discharged, but was readmitted November 25, 1991 because of fever and fatigue. A second course of OKT3 was administered from November 27 to December 6, 1991, after which the liver transaminases were found to increase instead of decrease. The fever persisted and a liver biopsy performed at that time showed massive infiltration with immunoblasts, while the heterophile agglutinins that were reported as negative on November 27 and December 2, 1991 became positive (1/896 before adsorption, 1/7 after boiled beef red cell adsorption, and 1/896 after guinea-pig kidney adsorption) on December 9, 1991. Because the liver donor was EBV and CMV seronegative, it was suspected that this patient might have been infected by transfused blood or by a natural route. Fever persisted until January 14, 1992, despite intravenous ganciclovir and solumedrol in addition to immunun and cyclosporine. The liver transaminases remained elevated (111/151). At this time, the patient also developed parotitis, a condition suspected to be etiologically associated with EBV. A throat wash that was negative for EBV on December 10, 1991 by culture and PCR, became culture- and PCR-positive for EBV on February 26, 1992. Liver biopsies continued to show lymphocytic infiltration and perivenous edema, and were positive for EBV by PCR from December 11, 1991 to March 2, 1992. Fatigue persisted until the end of March 1992, when liver transaminases finally returned to normal levels.

Patient no. 17. This infant girl with congenital biliary atresia and cirrhosis underwent liver transplantation on April 24, 1992, at the age of 9 months. The liver donor was EBV and CMV seronegative. The infant had been shown to be EBV and CMV seronegative at the age of 5 months. After transplant surgery, she was placed on CMV hyperimmune globulins. Within 1 week of surgery, she developed fever with tachypnea and pulmonary infiltrates, followed by multiple episodes of anemia and thrombocytopenia, without evidence of disseminated intravascular coagulation. She became CMV IgM+ on April 30, 1992; this suggested transmission of infection by a natural route or via transfused blood administered during the month preceding the transplantation because of bleeding esophageal varices. The urine sample obtained on May 9, 1992 was culture-positive for CMV and the lymphocyte culture of May 15, 1992 was culture-positive for EBV. Liver biopsies showed massive infiltration of portal triads with a polymorphic population of B lymphocytes; the presence of nodular hepatic lesions was shown by echography. Immunosuppressive regimens included immuran (33 days), cyclosporine (38 days), solumedrol (38 days), as well as antithymocyte globulin (ATG) (9 days), followed by one cycle of OKT3 (8 days). She remained febrile until death on June 1, 1992, but blood cultures remained negative, except for Staphylococcus epidermidis, which was positive at the end of May 1992. At autopsy, multiple liver nodules, as well as mediastinal and mesenteric lymph node tumors, were discovered, all of which showed the histologic features of polymorphic B-cell lymphomas. The liver biopsy, taken 10 days before death, was positive for EBV by the PCR technique.

Burden of EBV-infected cells in PB of transplant patients. Seven seronegative patients developed primary infection within the first 3 months posttransplant (Table 1). An additional patient (no. 8) was EBV-seronegative when tested at day 0 (ie, day of transplant). Lymphocyte culture of this same blood sample gave rise to EBNA+ lymphoblastoid cell lines, confirming primary EBV infection just before trans-
The burden of EBV-infected lymphocytes in the PB was generally greater in the group of transplant patients who underwent primary infection posttransplant compared with those who were already infected with EBV before transplant (Fig 1, Table 2). The number of EBV-transformed cells from patients who became infected posttransplant was particularly high at 3 and 6 months posttransplant. Thus, at 3 months the lower and upper limits were 1 and 1,300 cells per million, respectively; at 6 months these figures were 10 and 2,000 cells per million, respectively. By 1 year posttransplant, the titer fell to between 3 and 50 transformed B cells per million.

Individuals who were EBV+ pretransplant also showed higher numbers of EBV-infected cells in their PB after immunosuppressive therapy. However, the upper limit, attained at 6 months posttransplant, was 100 cells per million which, compared with the upper limit of 2,000 for EBV- pretransplant patients, represented a 20-fold difference between the two populations (P < .0005).

Statistical analysis of the data showed a significant difference between the EBV+ and EBV- groups (P < .044) at 3 months posttransplant. It is noteworthy that the highest increase in the number of EBV-infected lymphocytes at 3 months posttransplant was seen in a patient who developed fatal PTLD (patient no. 4) and in another with protracted infectious mononucleosis (patient no. 6). At 6 months the difference increased dramatically (P < .0005) and at 12 months the difference was not statistically significant (P < .267). The second analysis of variance for repeated measurements on 12 subjects showed an overall significant difference between subjects (P < .01); however, a highly significant difference between groups was noted (P < .005). The effect of sampling time, ignoring the groups, was significant only as a quadratic difference (P < .025). The interaction between the effect of sampling time on a specific group was only significant for the EBV+ group (P < .01), whereas this interaction was not significant for the EBV- group. The latter finding was confirmed by the increase in transformed cells in the EBV- group observable at 6 months only.

Viral load in PB as determined by PCR. The PCR was used to amplify EBV DNA in PB lymphocytes. This method allowed a semiquantitative evaluation of the number of EBV-infected cells in PB. The results shown in Fig 2 con-

### Table 2. Number of EBV Transformed Cells per 10⁶ Mononuclear Cells in PB of 16 Liver, Kidney, and Heart Pediatric Transplant Recipients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Pretransplant</th>
<th>3 mos posttransplant</th>
<th>6 mos posttransplant</th>
<th>1 yr posttransplant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NA</td>
<td>250</td>
<td>670</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.2</td>
<td>13</td>
<td>2,000</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.2</td>
<td>1</td>
<td>1,200</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.2</td>
<td>1,300*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.3</td>
<td>2</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>&lt;0.2</td>
<td>1,300†</td>
<td>100</td>
<td>1,000</td>
</tr>
<tr>
<td>7</td>
<td>&lt;0.3†</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>57%</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Renal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&lt;0.2</td>
<td>2</td>
<td>5</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>10</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>11</td>
<td>&lt;0.2</td>
<td>33</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>&lt;0.3</td>
<td>6</td>
<td>167</td>
<td>1.8</td>
</tr>
<tr>
<td>13</td>
<td>&lt;0.3</td>
<td>1</td>
<td>0.3</td>
<td>67</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>&lt;0.2</td>
<td>1.4</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td>&lt;0.2†</td>
<td>.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>&lt;0.2</td>
<td>.2</td>
<td>100</td>
<td>8</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not available because patient died; ND, not done.
* Patient died with PTLD.
† Patient developed protracted infectious mononucleosis at 6 weeks posttransplant.
‡ Patient developed primary liver failure.
§ Patient developed primary EBV infection just before transplant, documented by positive lymphocyte culture at day 0; died day 5 posttransplant of liver failure.
EBV load in peripheral blood and risk of PTLD

![Graph showing EBV titers in saliva of transplant patients.](image)

**Fig 2.** Plot showing EBV titers in saliva of transplant patients. EBV titers in sequential saliva samples obtained from patients who were EBV+ (C) or EBV- (B) before transplant were determined. Statistical analysis of the data showed significance values of $\alpha < .473$, $\alpha < .168$, and $\alpha < .063$ for the EBV+ group relative to the EBV- group at 3, 6, and 12 months, respectively.

EBV burden in saliva of transplant patients. After EBV infection, all saliva samples obtained from transplant patients were positive for viral DNA, as evaluated using the PCR technique. By culture, 86% of the EBV+ patients pretransplant and 75% of those experiencing primary EBV infection excreted virus posttransplant. The salivary viral titer was shown to increase after transplant surgery (Fig 2). It was noted that the quantity of virus found in saliva, as well as the number of excretors in the pediatric transplant population at 3, 6, and 12 months posttransplant, were independent of the patient’s EBV serologic status at the time of transplant. Further, the EBV titer in saliva was found to vary considerably with sampling times in any given individual (data not shown) and bore no correlation to the burden of EBV in blood. Statistical analysis of variance showed that the only significant increase in salivary EBV titers occurred in the pretransplant EBV+ group at 12 months posttransplant ($\alpha < .053$).

Correlation of EBV-infected cell burden in PB with development of PTLD. The most spectacular expansion of the number of EBV-transformed cells in PB, occurring 3 months posttransplant, was detected by culture in the only two transplant patients who developed PTLD (Tables 1, 2, 3, and Fig 1). Both of these patients were negative for EBV pretransplant. One of them, a 1-year-old infant (patient no. 4) died 3 months posttransplant after the development of polymorphous B-cell lymphoma in the transplanted liver. The other, a 17-year-old girl (patient no. 6), developed a protracted infectious mononucleosis syndrome 5 weeks posttransplant. A third PTLD (patient no. 17 in Table 3) occurred in a 12-month-old liver transplant patient after completion of this study.

**Discussion**

Examination of the immunosuppressive protocols showed that cyclosporine, immuran, and corticosteroids (solnemedrol/ prednisone) were used for all organ transplant groups, with the exception that renal transplant patients were given antilymphocyte globulin/ATG for the first 10 days after renal transplantation, which was then replaced by cyclosporine. Six out of a total of eight liver transplant patients received OKT3 (Table 3); however, none of the renal and only one cardiac transplant patient in our study received this drug. The latter was given OKT3 for a 12-day course at the end of the first 3 months after transplantation. Therefore, with the possible exception of OKT3, the immunosuppressive regimens of patients who did not develop PTLD did not seem to differ remarkably from the regimens of transplant patients who did develop PTLD (Table 3).

This work has shown that primary EBV infection or reactivation occurring in the context of the immunosuppressed transplant patient may lead to an increase in the number of EBV+ cells in PB. This B-cell expansion was most dramatic when primary EBV infection occurred while the patient was immunosuppressed. It is noteworthy to compare the results presented here for transplant patients with those of Rocchi et al. who reported that normal EBV-seropositive individuals had an EBV-infected B-cell burden of 1 in $10^6$ to 1 in $10^7$ total PB mononuclear cells. Patients in the acute phase of infectious mononucleosis showed approximately 1 infected B cell per $2 \times 10^3$ mononuclear cells. The latter corresponds roughly to the number we observe in our EBV+ pretransplant population after infection by EBV.

Among the 16 patients studied, 2 developed PTLD. Both of these yielded the highest count of EBV-infected cells in PB detected by culture at 3 months posttransplant. Both patients were EBV+ pretransplant. One of these patients (no. 4) developed PTLD 3 months posttransplant and died; the other (no. 6) recovered after experiencing protracted infectious mononucleosis 6 weeks posttransplant. Both patients gave blood and saliva cultures positive for EBV 3 months posttransplant.

The results reported here confirm those of other investigators who observed that transplant patients undergoing primary EBV infection posttransplant ran a higher risk of developing PTLD compared with patients who were EBV+ before transplant. In the 16 patients studied, no EBV-associated PTLD was detected in the group who was EBV+ before transplant. It follows that pediatric transplant patients are...
more at risk for developing PTLD, because approximately 50% of them are negative for EBV pretransplant, compared with 10% of adult patients. At 6 months posttransplant, the number of EBV-transformed cells detectable by culture was not predictive of PTLD risk in our small patient population.

Salivary viral titers were shown to rise posttransplant in 8 of 9 patients independently of their serologic EBV status before transplant. Thus, immunosuppression leads to a general increase in the salivary EBV titer, as also reported by other investigators. Further, it was found that quantity of virus detected in saliva varied considerably between sampling times in a given individual. No correlation was shown to exist between viral titers in blood and saliva in any given patient. Also noteworthy in this regard is the published observation that use of antivirals for prophylaxis or treatment of patients with herpesvirus infections diminished EBV shedding in the oropharynx, but had little effect on the EBV-infected B-cell burden in PB.

A recent study investigated quantitative oropharyngeal excretion of EBV over a 1-year posttransplant period in 23 cardiac and 23 renal transplant patients. These investigators, as we have also, noted, as we have also, that serology does not necessarily reflect severity of outcome. In fact, their 7 patients who developed PTLD had the lowest serologic responses to VCA and EA. Their results also showed that patients with primary infection shed higher amounts of virus in saliva compared with patients who were seropositive before transplant. Patients on acyclovir or ganciclovir treatment were excluded from their analysis. Our results reported in the present study

Table 3. Immunosuppressive and Antiviral Treatment Administered From Time of Transplant to 3 Months Posttransplant in Patients With PTLD and Matched Controls

<table>
<thead>
<tr>
<th>Age</th>
<th>Patient 6</th>
<th>Patient 1</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 17</th>
<th>Patient 8</th>
<th>Patient 2</th>
</tr>
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<tbody>
<tr>
<td>Cyclosporine</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>38 ds</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
</tr>
<tr>
<td>Immuran</td>
<td>64 ds*</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>21 ds*</td>
<td>&gt;93 ds</td>
<td>38 ds</td>
<td>1 d</td>
<td>60 ds*</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>&gt;93 ds</td>
<td>58 ds*</td>
<td>&gt;93 ds</td>
<td>38 ds</td>
<td>5 ds</td>
<td>&gt;93 ds</td>
</tr>
<tr>
<td>OKT3</td>
<td>20 ds (2)</td>
<td>11 ds</td>
<td>0</td>
<td>34 ds (3)</td>
<td>16 ds</td>
<td>8 ds</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALG/ATG</td>
<td>11 ds</td>
<td>11 ds</td>
<td>8 ds</td>
<td>1 d</td>
<td>10 ds</td>
<td>9 ds</td>
<td>4 ds</td>
<td>23 ds</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>10 ds</td>
<td>0</td>
<td>14 ds</td>
<td>10 ds (2)</td>
<td>24 ds (3)</td>
<td>1 d</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42 ds (3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>Primary EBV/CMV Infection</td>
<td>EBV only</td>
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<td>EBV only</td>
<td>Both</td>
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<td>EBV only</td>
</tr>
<tr>
<td>Outcome</td>
<td>PTLD, survived</td>
<td>Survived</td>
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<td>PTLD, died</td>
<td>Survived</td>
<td>PTLD, died</td>
<td>Died</td>
<td>Survived</td>
</tr>
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</table>

Patients 1, 3, 6, 8, and 2 were matched controls. Parentheses indicate number of cycles.
Abbreviations: ATG, antithymocyte globulin; ALG, antilymphocyte globulin.
* Interruption of medication to lessen immunosuppression.

Fig 3. Representative blot obtained from PCR-amplified DNA in PB of patients who were EBV- before transplant and who seroconverted shortly after transplantation. Blood was drawn before and at 3, 6, and 12 months posttransplant. Mononuclear cells were banded on Ficoll-Hypaque (Pharmacia Biotech Inc, Baie d'Urfe, Quebec, Canada) density gradients. The cells were washed, boiled, and an amount of DNA equivalent to 20,000 cells per reaction tube was amplified. A 304-bp segment within the BamHI-M region of the EBV genome was chosen for amplification. The top panel shows an autoradiograph after Southern blotting with an EBV-BamHI-specific oligoprobe. The bottom panel depicts the ethidium bromide-stained gels from which the Southern blots were derived. Note the presence of the 242-bp HLA-D& DNA marker band. The 500-bp band in the second lane of each block is amplified A phage DNA. Appropriate positive (Raj) and negative (WA-B, cord blood lymphocytes, H2O) controls were also included.
showed a significant difference between the groups only at 12 months posttransplant (α < .053), when the EBV+ pretransplant group shed more salivary virus. The difference between the data may be caused by the fact that we used cell culture to quantitate viral excretion (PCR was also used, but only as a semiquantitative tool), whereas Preiksaitis et al10 used a quantitative dot blot hybridization assay. Our results show unequivocally that transplant patients with primary EBV infection have, as a group, a 10- to 100-fold higher burden of EBV-infected cells in PB than do patients who were EBV+ before transplant. This higher load is most obvious at 3 months (P < .044) and 6 months (P < .0005) posttransplant and diminishes by 12 months (P < .269) posttransplant. Because both patients who developed PTLD had some of the highest amounts of EBV-infected cells at 3 months posttransplant, one might conclude that patients with greatly increased levels of circulating EBV-infected lymphocytes early after transplantation have a higher risk of developing PTLD, particularly if undergoing simultaneous primary cytomegalovirus (CMV) infection (Table 3). Sainte-Justine Hospital records to date show that the combination of primary EBV and CMV infections within 3 months of transplantation occurred in 4 of 9 transplant patients who died, but in only 1 of 54 who survived (P < .0009).36 Two of the 4 developed fatal EBV+ PTLD within 4 months of transplantation with donors by CMV- and EBV-seropositive donors.

The epithelial cells of the oropharynx constitute a productive system to EBV replication.2,3,27 Defective viral particles may ensue because of errors of replication of the viral DNA. Defective strains may manifest more difficulty to immortalize B cells and therefore, may not be as easily detectable by culture compared with their wild-type parental strains. In fact, a natural EBV variant, known as EBV type B, showed reduced transformation potential compared with type A.38 Viral variants are frequently found in the oropharynx39,40 and occasionally in blood,41 but their detection has been hampered because the conventional culture method is routinely used to test for the presence of EBV. For this reason, PCR amplification followed by Southern blotting of the amplified fragments, may be more suitable for detecting strains which do not readily immortalize lymphocytes in culture.

Finally, although quantitative lymphocyte culture from blood is accurate, relatively easy to perform and cost efficient, results using this method cannot be made available to the treating physicians before a minimal delay of 2 weeks. The PCR data presented here shows that this technique performed on serial dilutions of PB mononuclear cells, although costly and technically cumbersome, might constitute an ideal quantitative method, requiring a maximum of 3 days to yield results.

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Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of pediatric transplant patients and risk of lymphoproliferative disease

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