ORIGINAL ARTICLE

Immunohistochemical Analysis and Epstein-Barr Virus in the Tonsils of Transplant Recipients and Healthy Controls

Sarah E. Mowry, MD; Ali M. Strocker, MD; Jessica Chan, MD; Christopher Takehana, BS; Nader Kalantar, MD; Sunita Bhuta, MD; Nina L. Shapiro, MD

Objective: To compare lymphocyte immunohistochemical markers and staining for Epstein-Barr virus (EBV) in tonsillectomy specimens from healthy children and pediatric transplant recipients.

Design: Analysis of pathology specimens.

Setting: Tertiary care medical center.

Patients: Consecutive sample of tonsillectomy specimens from 60 pediatric solid organ transplant recipients and 60 healthy children.

Intervention: Immunohistochemical staining of tonsillectomy specimens for κ and λ light chains, B and T lymphocytes, EBV-encoded small nuclear RNA (EBV-EBER), and EBV-encoded latent membrane protein (EBV-LMP).

Main Outcome Measure: Detection of a difference in EBV activity in transplant recipients vs healthy controls.

Results: There was 1 case of posttransplantation lymphoproliferative disorder (PTLD). All other tonsillectomy specimens from both groups demonstrated follicular hyperplasia. Tonsillectomy specimens from both groups were polyclonal, expressing κ and λ light-chain activity, including the case of PTLD. The number of specimens staining positive for CD3 activity, a marker of T lymphocytes, was reduced in the transplant group (85%), compared with 100% in the control group ($P<.01$). EBV-EBER is a nuclear stain indicating active EBV infection, whereas EBV-LMP staining denotes latent infection. Twenty-seven of 60 transplant specimens (45%) demonstrated EBV-EBER activity compared with 0 of 60 control specimens ($P<.001$). EBV-LMP activity was equal in both groups.

Conclusions: Adenotonsillar hypertrophy in transplant recipients with no prior exposure to EBV may be a sign of active EBV infection. A high incidence of EBV-EBER was found in the tonsils of transplant recipients. Active adenotonsillar EBV infection in the setting of T-lymphocyte suppression in transplant recipients may be a potential early precursor of PTLD.

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Author Affiliations: Division of Otolaryngology–Head and Neck Surgery (Drs Mowry, Strocker, Kalantar, and Shapiro and Mr Takehana) and Department of Pathology and Laboratory Medicine (Drs Chan and Bhuta), David Geffen School of Medicine, University of California, Los Angeles.

POSTTRANSPLANTATION LYMPHOproliferative disorder (PTLD), a spectrum of lymphoid dysplasias, affects 2% to 7% of patients after solid organ transplant.[1] This disease is due to polyclonal immortalization of Epstein-Barr virus (EBV)-infected B lymphocytes in more than 80% of patients. Approximately 12% to 15% of PTLD cases are the result of T-cell proliferation.[2] The mildest form of lymphoproliferative disorder is polyclonal lymphocyte proliferation, while the other end of the spectrum is aggressive large cell lymphoma. Early-stage lesions typically respond to decreased levels of immunosuppression. However, patients who develop PLTD within the transplanted organ or those who develop monoclonal lymphomas have substantially poorer outcomes. PTLD-related lymphoma still carries a 40% to 50% mortality rate.[3]

The incidence of this potentially life- and organ-threatening disease is higher in children, with overall rates ranging from 4% to as high as 15%.[1,4] Children are thought to be at a higher risk for the development of PTLD as a result of higher rates of EBV seronegativity at the time of transplantation.[5] In immunocompetent patients, EBV infection is kept in check by $CD8^+$ cytotoxic T cells. However, transplant recipients receive potent medications to decrease cytotoxic T-cell activity and thereby prevent graft rejection. In these immunosuppressed patients, EBV-mediated B-cell proliferation proceeds unchecked, resulting in lymphoproliferative disorders.

PTLD can develop in numerous places in the body, including the transplanted organ. The Waldeyer ring is frequently involved in children. Epstein-Barr infection in immunosuppressed hosts may lead to EBV-related associated lymphoid hy-

perplasia, which is a known precursor to PTLD.[5] Adenotonsillar hypertrophy may be an early sign of abnormal B-cell proliferation in the transplant population, particularly in cases involving children with normal-appearing tonsillar tissue before transplantation. Other signs and symptoms of both acute EBV infection and PTLD are fever, prostration, hepatosplenomegaly, cervical lymphadenopathy, diarrhea, and poor appetite.

There are a variety of ways to detect EBV. In this study, we chose to investigate the expression of certain viral proteins and RNA using immunohistochemical (IHC) and in situ hybridization (ISH) techniques. During active infection, viral replication results in production of EBV-encoded small nuclear RNA (EBV-EBER). The virus can remain dormant for many years after the primary infection. During this latent infection, the protein profile changes, and EBV-encoded latent membrane protein (EBV-LMP) is used to detect the virus. Current views in the literature propose that PTLD results from primary active infection of transplant recipients.[1] Therefore, we decided to study the expression of B- and T-cell markers and EBV antigens in surgical adenotonsillar specimens both in healthy children and in children who had undergone solid organ transplantation. We hypothesized that children who had undergone solid organ transplantation would have higher rates of EBV infection, both latent and active.

METHODS

The institutional review board for human subjects at the University of California, Los Angeles, approved this study (UCLA IRB 05-09-110-02). A retrospective review of case files from the senior author's (N.L.S.) practice was conducted. Patients eligible for the study included those referred from the Mattel Children's Hospital UCLA liver transplantation and kidney transplantation programs for adenotonsillectomy for any reason. Data were also obtained from healthy controls undergoing adenotonsillectomy for sleep-disordered breathing, chronic tonsillitis, or recurrent otitis media. Patient records were reviewed for age at time of operation, sex, type of operation performed, and indications for operation. Patients who had undergone transplantation were reviewed for age at transplantation, type of transplantation, and need for subsequent transplantation.

Tissue specimens obtained at surgery were sent as fresh specimens. The pathologic diagnosis of the tonsil and adenoid specimens was made using standard histologic and IHC techniques. Tissue samples were classified based on the Society of Hematopathology Workshop 1997 recommendations for PTLD.[6] The virus was detected by ISH for EBV-EBER and EBV-LMP. Clonality of the B-cell proliferation was identified using IHC staining of the B-cell–associated κ and λ light chains. Presence of both κ and λ light chains indicated polyclonal proliferation. The IHC techniques were also used to detect standard T- and B-cell markers, CD3 and CD20, respectively. The results were reviewed with a senior pathologist (S.M.B.). The groups were compared using the paired *t* test.

RESULTS

One hundred twenty children who had undergone surgery from September 1999 to June 2005 were identified for the study. Sixty patients had undergone solid organ transplantation: 36 (60%) had received liver transplants, 4 (7%) had received heart transplants, and 20 (33%) had received kidney transplants. Four of the liver transplant recipients had 2 grafts, and 1 of the kidney transplant recipients had undergone a second transplantation because of graft failure. There were 28 female and 32 male transplant recipients. Their ages ranged from 2 to 19 years (mean age, 8.6 years; median age, 8.0 years) at the time of surgery. Fifty-two of the 60 patients underwent surgery to rule out PTLD after manifesting adenotonsillar hypertrophy. The remaining patients underwent surgery because of recurrent tonsillitis, tonsillar bleeding, recurrent otitis media, or sleep apnea. Three patients underwent tonsillectomy alone; 1 patient underwent adenoidectomy alone.

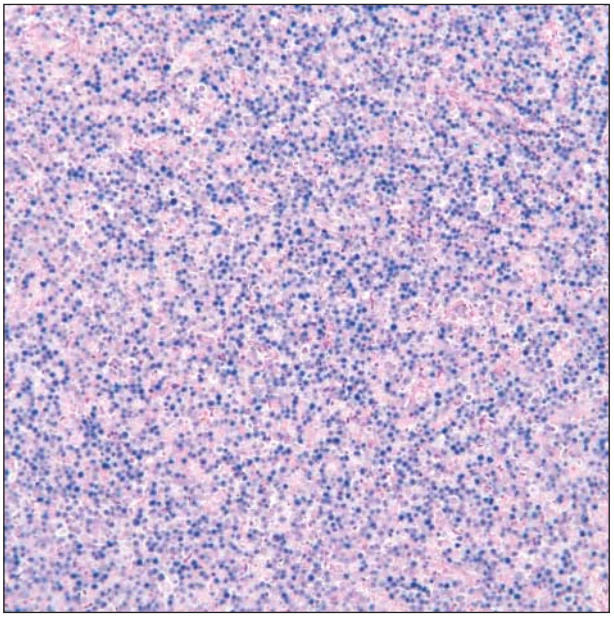

Figure 1. Tonsillar tissue from the patient with posttransplantation lymphoproliferative disorder. Note the proliferation of B cells with prominent nucleoli (hematoxylin-eosin, original magnification ×200).

Sixty control patients were enrolled as well. Thirty-two were female and 28 were male. Their ages ranged from 18 months to 19 years (mean age, 6.0 years; median age, 5.0 years) at the time of surgery. Indications for operation in this group were sleep-disordered breathing, chronic tonsillitis, peritonsillar abscess, or chronic otitis media. One patient underwent adenoidectomy alone; the remainder of the cohort underwent adenotonsillectomy.

Of the transplant recipients, 1 was diagnosed as having PTLD and 20 demonstrated immunohistologic evidence of EBV-related lymphoid hyperplasia (**Figure 1**). The specimens from all other patients demonstrated reactive lymphoid hyperplasia with no evidence of PTLD on routine analysis. All control specimens demonstrated reactive follicular hyperplasia (**Figure 2**). Evaluation of clonality of specimens via expression of κ and λ light chains demonstrated that all hyperplasia was polyclonal, including the case of PTLD. There was a significant difference in the presence of T cells, represented by CD3 stain, a T-cell marker. While 100% of the healthy controls demonstrated T-cell activity in the specimens, only 84% of transplant recipients had detectable CD3 activity on IHC staining ($P < .01$).

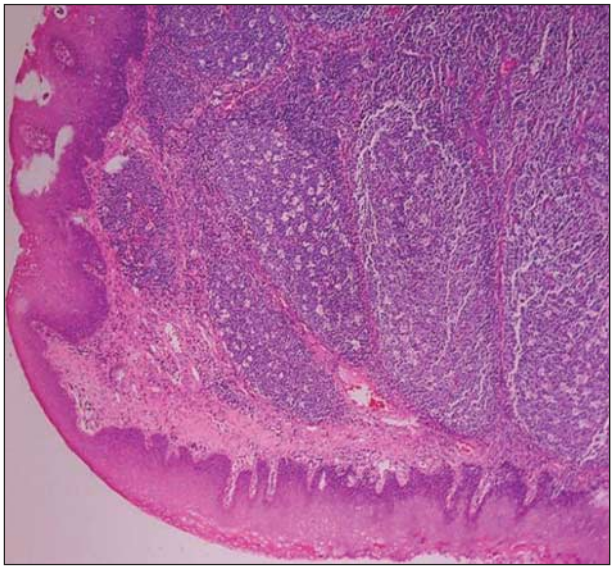

Figure 2. Normal lymphoid hyperplasia. Note the germinal centers and starry sky appearance (hematoxylin-eosin, original magnification ×20).

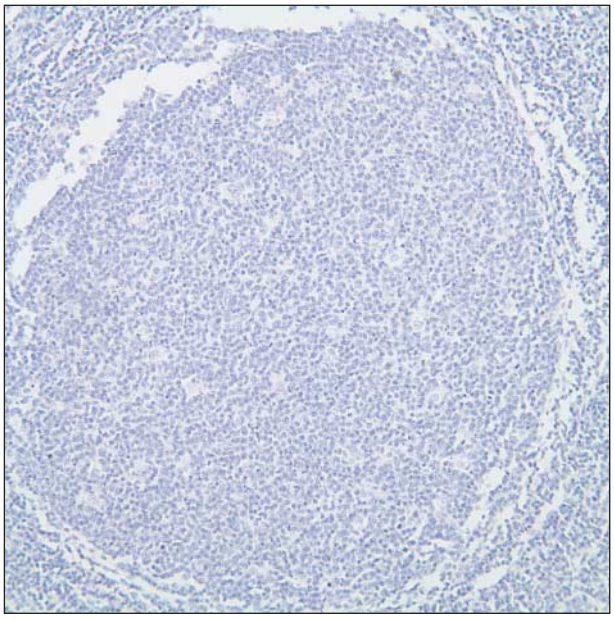

Figure 3. Immunohistochemical stain for Epstein-Barr virus–encoded small nuclear RNA in a specimen from a healthy control. Like specimens from all healthy controls, this specimen demonstrates no reactivity (hematoxylin-eosin, original magnification ×200).

Results of EBV studies demonstrated significant differences between transplant recipients and controls: EBV-EBER activity was present in 27 of 60 transplant specimens (45%) compared with 0 of 60 controls ($P < .001$) (**Figure 3** and **Figure 4**); EBV-LMP activity was equal in both groups (**Table**). The patient diagnosed as having PTLD demonstrated strong reactivity for EBV-EBER and EBV-LMP in both small and large cells.

COMMENT

Although many transplant centers have instituted protocols for prolonged antiviral therapy for EBV-seronegative patients receiving EBV-positive grafts, EBV infection can still occur.[4] In a small percentage of patients, this infection can lead to PTLD. The transplant recipients in this study demonstrated high rates of active EBV infection and decreased T-cell activity when compared with healthy controls. One patient in the study had polyclonal PTLD and active EBV infection.

Adenotonsillar hypertrophy is common in children. However, in immunosuppressed children, this tissue enlargement may herald more ominous pathology. The true incidence of adenotonsillar hypertrophy in this susceptible population is not known. Previously, Shapiro and Strocker[7] demonstrated that patients who were EBV seronegative at the time of transplantation had larger tonsils and adenoids than their seropositive counterparts. This finding was confirmed in several follow-up studies.[8,9] The implication is that these patients are at higher risk to develop some form of PTLD. Several other authors have described PTLD arising in the adenotonsillar tissue.[10,11] Herrmann et al[10] reported that 25% to 39% of patients with PTLD present with head and neck manifestations, regardless of the transplanted organ. Broughton et al[11] reviewed the pathologic findings in cases involving children who had undergone tonsillectomy after

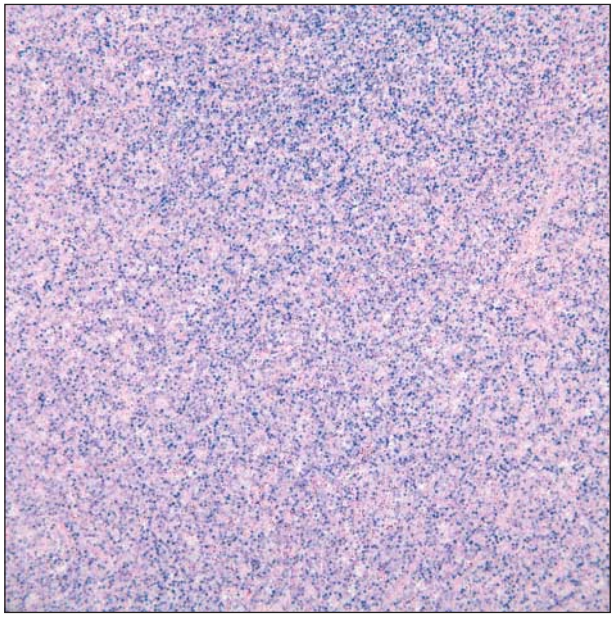

Figure 4. Immunohistochemical stain for Epstein-Barr virus–encoded small nuclear RNA in a specimen from a patient with posttransplantation lymphoproliferative disorder. Note the strong reactivity within the nucleus of the infected cells (hematoxylin-eosin, original magnification ×200).

Table. Immunohistochemical and In Situ Hybridization Results

Group	EBV-EBER Positive[a]	EBV-LMP Positive[b]
Transplant recipients	27/60	5/60
Controls	0/60	6/50

Abbreviations: EBV-EBER, Epstein-Barr virus–encoded small nuclear RNA; EBV-LMP, EBV-encoded latent membrane protein.
[a] $P < .001$.
[b] $P = .95$.

transplantation and found that 40% had PTLD-related changes. Collectively, these groups concluded that any change in a child's head and neck examination with regard to the lymphoreticular system should prompt immediate further investigation and surgical intervention.

Part of our cohort has been previously described.[12] In that study, Williamson et al[12] reported the histologic, IHC, and ISH results in 21 of the 60 transplant cases in the current study. In the previous study, all specimens had tested positive for EBV-EBER (12 of 12 specimens tested). With the larger cohort, we have demonstrated that EBV-EBER and EBV-LMP activity is not ubiquitous in this vulnerable population. However, transplant recipients have high rates of active infection. Twenty-seven of the current cohort (45%) demonstrated EBV-EBER activity on ISH studies. It is the practice at our institution to treat such patients with a high index of suspicion for PTLD for this reason and strongly recommend adenotonsillectomy in immunosuppressed patients with adenotonsillar hypertrophy.

To our knowledge, this is the first study to compare EBV activity between transplant recipients and healthy control subjects. Only 10 of 60 healthy control subjects (16%) demonstrated latent infection with EBV (EBV-LMP), and none demonstrated active infection (EBV-EBER). These findings are consistent with classic epidemiological data on EBV infection reporting that approximately 40% of the 8- to 10-year-old patients were seropositive for EBV.[13] The mean age of the control subjects in this study was 6 years; therefore, lower rates of seropositivity are to be expected. Four of the 60 transplant recipients demonstrated latent infection but significantly more demonstrated active EBV infection. Nearly half of the transplant recipients demonstrated staining for EBV-EBER. This difference was statistically significant ($P = .01$). Because PTLD is a spectrum of disease that results from active EBV infection in the majority of affected patients, these findings lend credence to the recommendation that these patients should undergo adenotonsillectomy and that consideration should be given to lowering the immunosuppressive dosages.

There are several limitations to our study. First, the patients' pretransplantation EBV status was not evaluated. Therefore, it is unclear whether the active infection seen in these patients was primary or reactivation of latent disease. Second, this study was a retrospective pathologic review. Epstein-Barr titers were not correlated with pathologic findings, which would help indicate the severity of the infection and the risk for the development of PTLD. Finally, the transplant recipients' level of immunosuppression was not correlated with EBV activity. It is likely that increasing levels of immunosuppressive medications would increase the number of active EBV infections. However, it is unclear whether the level of immunosuppression would affect the number of latent EBV infections.

In conclusion, pediatric transplant recipients have significantly higher rates of active EBV infection than healthy controls. These children are at increased risk of developing EBV-related PTLD as a result of their immunosuppressive medications. Of the children who were referred to rule out lymphoproliferative disorders, nearly half demonstrated PTLD-related changes and 1 had frank PTLD. Frequent long-term surveillance of this population is necessary. When changes are noted, immediate surgical intervention should be strongly considered.

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Correspondence: Nina L. Shapiro, MD, Division of Otolaryngology–Head and Neck Surgery, David Geffen School of Medicine, UCLA Box 951624, 62-158A CHS, Los Angeles, CA 90095-1624 (nshapiro@ucla.edu).
Author Contributions: Dr Mowry had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Strocker, Bhuta, and Shapiro. *Acquisition of data:* Strocker, Chan, Takehana, Kalantar, Bhuta, and Shapiro. *Analysis and interpretation of data:* Mowry, Chan, and Bhuta. *Drafting of the manuscript:* Mowry. *Critical revision of the manuscript for important intellectual content:* Mowry, Strocker, Chan, Takehana, Kalantar, Bhuta, and Shapiro. *Obtained funding:* Shapiro. *Administrative, technical, and material support:* Chan and Bhuta. *Study supervision:* Strocker, Bhuta, and Shapiro.
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Previous Presentation: This study was presented in part at the 2007 Annual meeting of the American Society of Pediatric Otolaryngology; April 27-29, 2007; San Diego, California; and was awarded third place in the basic science division.
Additional Contributions: Itsushi Peter Shintaku, PhD, assisted in the IHC analysis, and ArthroCare provided support to the Department of Pathology and Laboratory Medicine in the IHC stain processing.

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