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Case report

Detection of EBV DNA in the cord blood donor for a patient developing Epstein–Barr virus-associated lymphoproliferative disorder following mismatched unrelated umbilical cord blood transplantation

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Summary:

Epstein–Barr virus-associated post-transplant lymphoproliferative disorder (PTLD) has been well described as a complication following allogeneic stem cell transplantation but has only recently been reported following umbilical cord blood (UCB) transplant. We report the case of a child transplanted with unrelated mismatched UCB for juvenile chronic myelogenous leukemia (JCML) who developed EBV-associated PTLD, which was confirmed pathologically, 139 days following stem cell infusion. There was no clinical response to reduction of immune suppression, high-dose acyclovir, or alpha interferon. The patient died 160 days after transplantation. EBV was detected by polymerase chain reaction in the cord blood unit used for transplantation. This case demonstrates that EBV-associated PTLD can occur following mismatched unrelated UCB transplant and may be related to transmission of EBV infection by donor lymphocytes. *Bone Marrow Transplantation* (2001) **27,** 761–765.

Keywords: PTLD; umbilical cord blood; transplant; EBV

Epstein–Barr virus (EBV)-associated PTLD has been well described as a complication following allogeneic stem cell transplantation but has only recently been reported following UCB transplantation.[1,2] The overall incidence of PTLD in patients who undergo hematopoietic stem cell transplant varies with donor stem cell source, degree of HLA matching, type of graft-versus-host disease (GVHD) prophylaxis, primary underlying disease, and type of graft manipulation.[1] Patients with primary immunodeficiencies transplanted with an unrelated HLA mismatched, T cell-depleted graft are at the highest risk with a probability of developing EBV-associated PTLD that may be as high as 65%.[3] Umbilical cord blood has heretofore been considered a pristine source of EBV-uninfected stem cells. We describe the case of a child who developed EBV-associated PTLD following hematopoietic stem cell transplantation from an unrelated mismatched cord blood donor with evidence of EBV DNA in the cord blood, which was identified only after the child's death.

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Case report

A 29-month-old Hispanic boy with JCML at 19 months of age underwent stem cell transplantation with mismatched unrelated UCB 3 months after presentation. The UCB unit was an HLA-A and -B serologic match with a mismatch at the DR locus by high-resolution DNA testing. Viral serologic testing at the time of diagnosis of JCML revealed negative VCA-IgM and VCA-IgG EBV antibody titers, and positive IgG but negative IgM antibody titers to CMV indicating past infection. Repeat EBV serologies prior to transplantation, after blood and platelet transfusions, showed low levels of VCA-IgG, but no VCA-IgM. Serologic tests for varicella-zoster virus, hepatitis A, B, and C, and HIV were negative.

The conditioning regimen included total body irradiation 150 cGy twice daily for 4 days, thiotepa 10 mg/kg on day −6, etoposide 1000 mg/m^2 by continuous infusion over 24 h on days −6 and −5 (total dose 2000 mg/m^2), and cyclophosphamide 60 mg/kg once daily i.v. on days −4, −3, and −2 (total dose 180 mg/kg). GVHD prophylaxis consisted of anti-thymocyte globulin (ATG) 20 mg/kg i.v. once daily on days +1, +3, +5, and +7 with methylprednisolone 2 mg kg i.v.; methotrexate 15 mg/kg i.v. on day +1 and 10 mg/kg i.v. on days +3 and +6; and continuous infusion cyclosporin A 5 mg/kg/day i.v. beginning on day −1 to maintain levels of 300–400 ng/ml. White blood cell engraftment occurred on day +22, with platelet engraftment on day +47. Biopsy-proven grade I, stage II acute GVHD of the skin developed on day +18 and responded to methylprednisolone 2 mg/kg/day. The hospital course was notable for grade 2 mucositis, and bacteremia with two different coagulase-negative staphylococcal species and *Enterobacter cloacae*

that resolved with antibiotic therapy and removal of the central venous line. The patient was discharged home on day +27 at which point PCR analysis of variable number tandem repeat (VNTR) markers confirmed 100% donor cell engraftment.

The dose of corticosteroids was gradually tapered and discontinued by day +100. On day +122 the patient developed an acute upper respiratory tract infection treated with cefprozil, and an exacerbation of acute GVHD of the skin treated with prednisone 2 mg/kg/day. The patient was admitted to the hospital on day +126 because of low-grade fever, persistent rhinorrhea and cough, presumed candidal esophagitis, and dehydration. Chest radiograph showed an infiltrate (Figure 1a). Rapid antigen studies of nasal washings for RSV, parainfluenza, influenza, and adenovirus were negative. The GVHD of the skin progressed and the dose of corticosteroids was increased to 4 mg/kg/day for 4 days. The rash cleared and the patient improved. On day +131 the patient developed hepatosplenomegaly. Hepatic transaminases were mildly elevated, while coagulation studies and bilirubin were normal. Serology tests for acute infection with EBV, CMV, and hepatitis A, B and C were negative. Subsequently, the patient developed massive cervical lymphadenopathy with upper airway obstruction. Computed tomography scan demonstrated extensive lymphadenopathy in the neck and nasopharynx (Figure 1b). Lymph node biopsy showed a clonal B cell proliferation consistent with PTLD. The presence of EBV in the tumor was documented by immunohistochemical stains positive for Epstein–Barr virus latent membrane protein (LMP). A bone marrow aspirate revealed normal marrow elements and cellularity with a mild degree of hemophagocytosis. PCR analysis of VNTR markers performed on peripheral blood WBCs reconfirmed 100% donor engraftment.

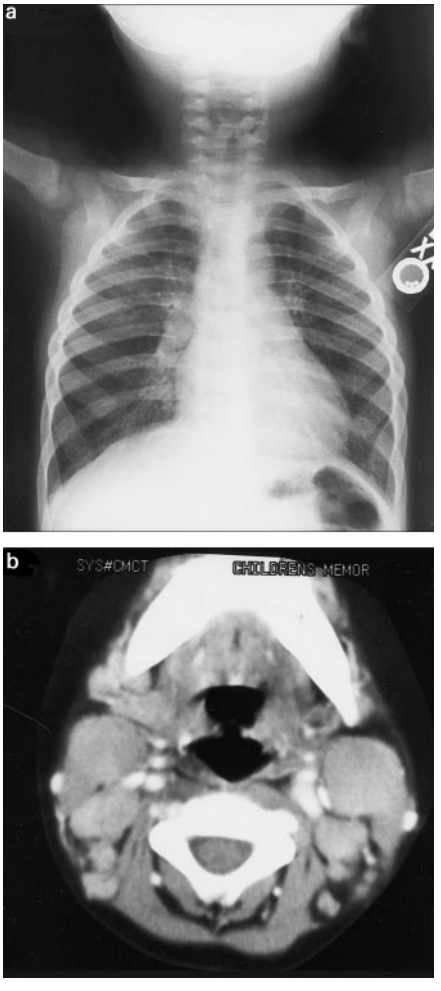

Figure 1 Chest radiograph. (**a**) The chest radiograph demonstrates fine interstitial opacities and hyperaeration. (**b**) Computed tomography scan of the neck. Computed tomography scan of the neck demonstrates bilateral enlarged cervical lymph nodes, later confirmed by biopsy to be post-transplant lymphoproliferative disease.

Immune suppression was tapered without resolution of the airway obstruction. The patient was electively intubated and a bronchoalveolar lavage (BAL) was performed to evaluate new infiltrates on the chest radiograph. GVHD remained quiescent. The patient developed worsening pulmonary disease and required oscillatory ventilation. Cultures from the BAL grew *Candida tropicalis*, *Blastoschizomyces capitatus* and *Enterococcus faecalis.* The patient received appropriate anti-infective therapy. Acyclovir (1500 mg/m^2/day) and alpha interferon (3 million units/m^2/day) were given to treat the PTLD. The patient deteriorated with progressive swelling of the head and neck and hepatosplenomegaly. Endotracheal cultures remained positive for *C. tropicalis*. On day +160 the decision was made to withdraw care. A post-mortem examination was performed.

Methods

Biopsy and autopsy specimens

Tissue from the lymph node biopsy was fixed in B5, processed, and stained for routine histologic examination. Tissues from the autopsy were fixed in formalin and processed similarly. Material from both the lymph node biopsy and the autopsy was snap-frozen in liquid nitrogen for DNA isolation.

Immunohistochemistry

Immunohistochemistry was performed on biopsy and autopsy specimens using a Ventana NexES Immunostainer (Ventana Medical Systems, Tucson, AZ, USA). Tissues were incubated with antibodies to CD20, CD45RO, kappa, lambda (Ventana Medical Systems) and EBV LMP (Dako, Carpenteria, CA, USA). Antigen binding was visualized using a DAB Detection Kit (Ventana Medical Systems).

Gene rearrangement studies

Snap-frozen tissue from the lymph node biopsy was used to extract DNA for PCR analysis of immunoglobulin heavy chain rearrangement. PCR was performed by using rapid cycling techniques previously described.[4] Rearrangements were sought using consensus FRIII and JH primers.[5] Beta-actin primers (Gibco BRL, Gaithersburg, MD, USA) were

also used to verify the presence of amplifiable DNA in each sample.

HLA typing of the tumor tissue

Snap-frozen tissue from the spleen and liver obtained at autopsy was used for high-resolution DNA typing of the DRB1 locus, performed by oligotyping with sequence-specific oligonucleotides and by gene sequencing.[6] Class II allele designations were based on DNA sequence variation.[7]

PCR for EBV

Total intracellular DNA was extracted from UCB cells using standard methods. PCR was performed using 0.67 μg of UCB DNA (equivalent 100 000 cells) and primers amplifying a 140 bp portion of the EBV *Bam*HI W region.[8] Primers PCO4 and GH20 amplifying a conserved β-globin sequence were included. A set of EBV copy-number controls consisting of 1–1000 copies of a linearized plasmid containing EBV *Bam*HI W diluted in a background of 10^5 lysed uninfected H9 cells was amplified simultaneously for semi-quantitation. Cycling conditions were 94°C (3 min) and 40 cycles of 68°C (10 min). Hybridization was performed using EBV *Bam*HI W.[7]

Results

Biopsy specimen

Hematoxylin and eosin-stained B5 fixed sections showed effacement of normal lymphoid architecture and replacement by a polymorphous infiltrate consisting of a mixture of plasma cells, intermediate and large lymphoid cells, and numerous immunoblasts. Numerous mitotic figures were identified; no areas of necrosis were seen in the biopsied tissue (Figure 2).

Immunohistochemical stains showed numerous $CD20^+$ cells confirming the B cell nature of the proliferation. No cytoplasmic immunoglobulin light chain restriction was identified. Virtually all B cells present showed cytoplasmic staining for EBV LMP.

PCR studies for immunoglobulin heavy chain rearrangement identified the presence of a clonal B cell population. There was insufficient tissue available for EBV clonality studies.

Autopsy specimen

At autopsy virtually all organs showed a B cell infiltration with similar morphology to that seen in the previously biopsied lymph node. There was particularly dense infiltration in the gastrointestinal tract, lungs, liver, spleen, and epiglottis. HLA typing performed on the affected cellular proliferation from the liver and spleen was compared to the known HLA typing from the patient and the UCB and demonstrated only the DRB1 0411 allele from the donor.

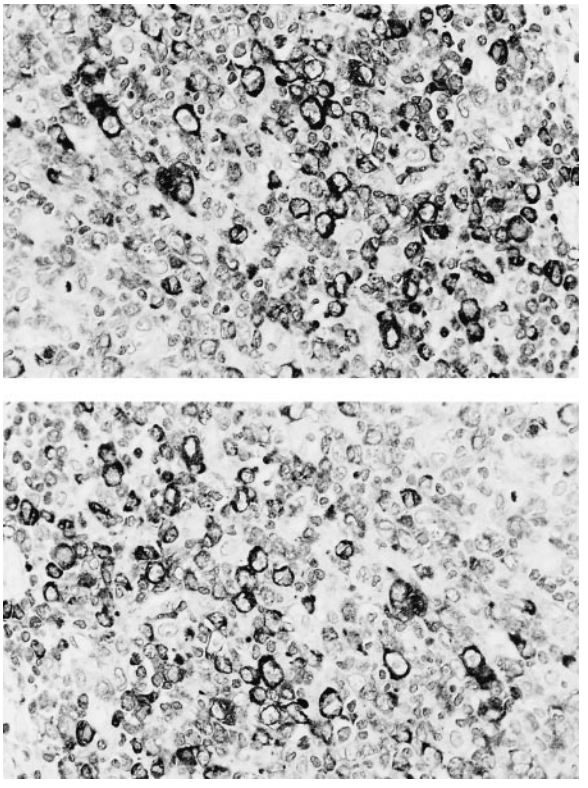

Figure 2 Lymph node biopsy specimen (hematoxylin and eosin). Effacement of normal architecture and replacement by a polymorphous infiltrate consisting of a mixture of plasma cells, intermediate and large lymphoid cells, and numerous immunoblasts.

Umbilical cord blood unit and mother's blood

The UCB and the mother's blood were tested and negative for the following: hepatitis B core antigen, hepatitis B surface antibody, anti-hepatitis C virus (HCV), human immunodeficiency virus (HIV) P24 antigen, anti-HIV I/II, anti-HTLV I/II, and RPR (for syphilis). Cytomegalovirus (CMV) IgG was positive on both the UCB unit and the mother's blood. The mother's CMV IgM serology was negative. Samples were not available for testing for EBV serology.

Testing for EBV by PCR was performed on a DNA sample from the UCB that had been stored at the time of original collection. Approximately 1 copy of EBV DNA was detected per 100 000 cells (Figure 3).

Discussion

PTLD is a well described entity following hematopoietic stem cell transplantation, with higher rates occurring in the setting of T cell depletion and/or greater degree of HLA disparity between donor and recipient.[1,9] We now report a second case of EBV-associated PTLD following unrelated UCB transplant. This case confirms that patients receiving a UCB transplant, at least in the unrelated, mismatched setting, are susceptible to this serious complication of stem cell transplantation. Our patient had several well-known risk factors for the development of EBV-associated PTLD

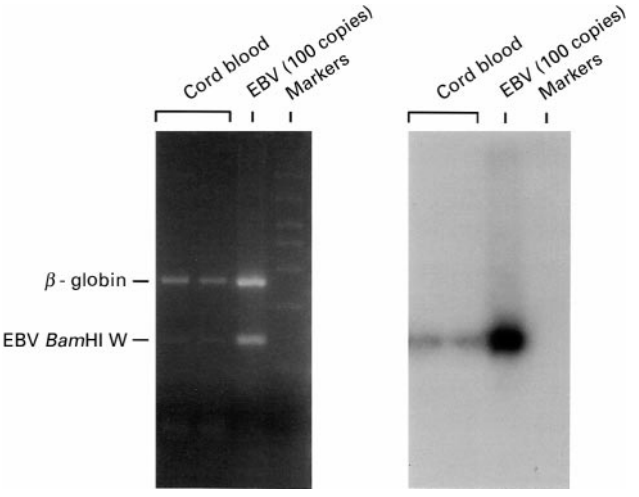

Figure 3 Results of PCR testing for EBV. Positive detection of EBV *Bam*HI in the cord blood cells at a sensitivity of one in 100 000 cells.

including an HLA mismatched stem cell source and the use of ATG for GVHD prophylaxis. In addition, the phenotypic and functional immaturity of T cells in a UCB graft might be akin to *in vitro* T cell depletion and increase the risk of developing PTLD.[10,11] Nevertheless, this patient was at extremely low risk for EBV-associated PTLD since he had not been infected with EBV prior to transplant, and UCB was used for donor cells.

Another factor that has been shown to be a predictor of PTLD is absence of prior exposure and immune response to EBV. Riddler *et al*[12] demonstrated that EBV burden in the peripheral blood following cardiac transplant is predictive of the development of PTLD, and that lack of EBV antibodies prior to transplant is a predictor of fatal outcome. Our patient was seronegative at the time of his diagnosis with JCML, but did have low VCA-IgG EBV antibodies at the pre-transplant evaluation, 4 weeks prior to the beginning of his conditioning regimen, from previous transfusion exposure. This low level of passively acquired VCA-IgG EBV antibody did not prevent the development of fatal PTLD.

Treatment for EBV-associated PTLD has not been rigorously evaluated. Approaches include reduction of immune suppression, high-dose acyclovir, ganciclovir, alpha interferon, cytotoxic drugs, B cell-specific monoclonal antibodies, and cytotoxic T cell therapy.[1] Reduction of immune suppression may result in the resolution of the PTLD, with up to a 40% response rate in renal transplant patients, but carries a significant risk of GVHD in the setting of hematopoietic stem cell transplant.[13] Cytotoxic chemotherapy may induce a remission in more than 50% of patients but carries risks that include graft failure.[14] Responses have been demonstrated in small series of patients utilizing alpha interferon, antiviral agents, or monoclonal antibodies.[15–19] More recent work with cytotoxic donor lymphocyte infusions shows great promise for the future.[20,21] Our patient did not respond to withdrawal of immune suppression, acyclovir, or alpha interferon. Chemotherapy was considered but was withheld due to the patient's active fungal infection and poor condition. Adoptive cytotoxic immunotherapy was not an option due to the lack of access to the donor.

The source of the EBV in most patients with PTLD has been identified as the donor.[22] In this particular case the tumor tissue was confirmed to be of donor origin by HLA typing, and as opposed to the case reported by Ohga *et al*,[2] EBV DNA was identified in the cord blood using PCR. Neither of these results provides conclusive evidence of the UCB as the source of the EBV infection in this case. However, the presence of EBV DNA in the UCB clearly demonstrates the potential for EBV transmission by UCB transplant. EBV DNA has been detected by nested PCR immediately after birth in the monocytes of two of 16 neonates born to HIV-infected mothers.[23] In this case the EBV burden in the UCB transplant used was low (~1 copy per 100 000 cells), consistent with the 1 in 10^6 latently EBV-infected lymphocytes found in persons with past EBV infection.[24]

No information or serum is available to determine the EBV infection status of the mother or the donor prior to transplant. It is most likely that the mother was latently infected with EBV since approximately 97.4% of pregnant women are seropositive, and seroconversion of EBV-seronegative women during pregnancy is uncommon, occurring in approximately 3.1% of EBV-seronegative women during gestation.[25] The presence of EBV in the UCB indicates potential transmission of EBV by donor lymphocytes, or alternatively by EBV-infected maternal lymphocytes, or as cell-free virus resulting from contamination of fetal blood with maternal blood prior to or during delivery. Andronikou *et al*[26] recently reported the death of a premature infant who was co-infected with EBV and CMV providing additional support for the perinatal acquisition of EBV.

While UCB is increasingly used as a source of hematopoietic stem cells for transplantation for both malignant and nonmalignant disorders, the relatively small number of transplants performed to date precludes complete understanding of the risks of this therapy. It is not surprising that EBV-associated PTLD can occur following UCB transplant, at least in the unrelated mismatched setting, and adds one additional complication that contributes to the morbidity and mortality associated with UCB transplant. With the lack of opportunity for adoptive immunotherapy as a treatment modality in the unrelated UCB transplant, screening for EBV, especially if a sensitive and reliable PCR test for the EBV genome is available, may play a role in the future evaluation of umbilical cord blood prior to use in transplantation.[27] However, the possibility of PTLD will always exist for EBV-seropositive transplant recipients. Development of *ex vivo* expanded EBV-specific cytotoxic T cells from UCB units could potentially be used to thwart this life-threatening complication.

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