Table 1
Enzyme activities in cerebrum and liver.

	IDS[a]	β-Hex[b]	β-Gal[b]
Cerebrum			
Control[c]	131.5	1141	71
Patient[c]	1.4	2135	44
Untreated MPS II[c]	0.5	1931	19
Liver			
Control (n = 3)	14.0–19.7	1144–1197	214–270
Patient[c]	6.3	1303	233
Fibroblast			
Control	88.7	Not done	Not done

[a] nmol/4 h/mg.
[b] nmol/h/mg.
[c] Average of duplicate measurements from different pieces of the tissue.

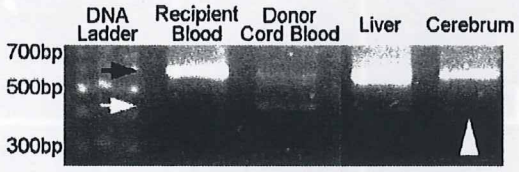

Fig. 4. VNTR analysis of MPS II post-CBSCT. The donor cord blood-derived band (about 450 bp, white arrow) can be distinguished from that of the recipient (pre-CBSCT). The brain of the patient after CBSCT, as well as the liver, seems to have the donor-derived band (white arrowhead) in addition to the recipient-derived band (about 600 bp, black arrow).

to its heterozygosity. Therefore, the presence of donor-derived cells was able to be confirmed by their lower band (about 450 bp; Fig. 4, white arrow). In addition to the concrete recipient-derived band which was detected in both liver and cerebrum of the patient after CBSCT, the weak donor-derived bands were detected in the liver and the cerebrum (Fig. 4, white arrowhead) of the patient at 10 months post-CBSCT, indicating that some donor cells had migrated into the liver and the cerebrum. The lower intensity of the donor-derived band in the cerebrum than in the liver suggested fewer donor-derived cells residing in the cerebrum.

3.4. Immunohistochemical study on IDS

Before performing immunohistochemical studies on IDS, we investigated the validity of the antibody. According to immunoblot analysis of IDS, the antibody reacted with recombinant human IDS having the predicted size of 76 kDa but not with recombinant human α-glucosidase as a negative control (Fig. 5A, arrow). Although by IDS immunoblot analysis the antibody did not react with normal human fibroblasts, probably because of low level of IDS in them (data not shown), immunocytochemical study revealed IDS-immunoreactivity in normal control fibroblasts (Supplemental method and Supplemental Fig. 2). According to immunohistochemical studies on unfixed-frozen cerebrum samples, IDS-immunoreactivity was positive in the non-MPS II control (Fig. 5B, arrows) but negative in the untreated MPS II (Fig. 5C). Furthermore, immunoreactivity of IDS (Fig. 5D, green arrows) and that of Lamp2 (Fig. 5E, red arrows), a lysosomal marker, were co-localized in the cerebrum of the non-MPS II control (Fig. 5F, yellow arrows). These results confirmed the validity of the antibody against IDS used in this study.

3.4.1. Distribution of IDS-positive cells in the liver

The routine avidin–biotin complex procedure used in the immunohistochemistry for the CNS did not work in the normal liver (data not shown), perhaps because the IDS enzyme activity

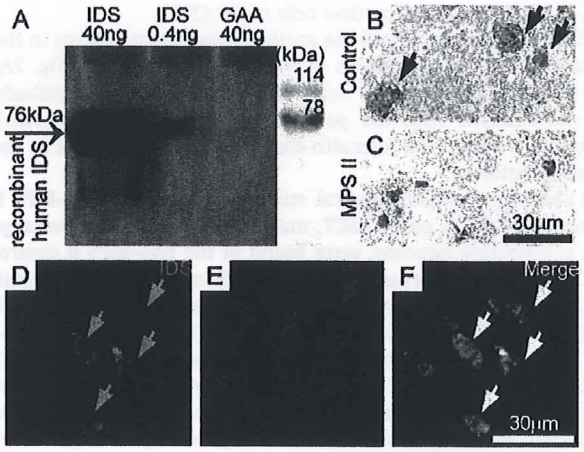

Fig. 5. (A) Immunoblot analysis of IDS. The antibody against IDS reacts specifically with recombinant human IDS having the predicted size of 76 kDa (arrow). GAA indicates recombinant human α-glucosidase, used as a negative control. (B and C) Immunohistochemical study on IDS in the cerebrum of non-MPS II control (B) and untreated MPS II (C). IDS-immunoreactivity is positive in the non-MPS II control (B, arrows) but negative in the untreated MPS II (C). (D–F) Double immunofluorescence for IDS and Lamp2, a lysosomal marker, in the cerebrum of the non-MPS II control. Immunoreactivity of IDS (D, green arrows) and that of Lamp2 (E, red arrows) are co-localized (F, yellow arrows). Blue fluorescence in "F" is from DAPI.

there was only about one-tenth of that in the normal cerebrum (Table 1). Therefore, the Tyramide Signal Amplification system was applied for the study of the liver.

IDS-immunoreactivity was detected in many cells of the MPS II post-CBSCT (Fig. 6A) as well as in the non-MPS II control (data not shown). Prior incubation of IDS antibody with excess antigen completely abolished the immunoreactivity, confirming the validity of the staining (Fig. 6B). In the double immunofluorescence staining for IDS and CD68 (Fig. 6C–E), IDS-immunoreactivity was detected in many cells (Fig. 6C, green arrows), not only in CD68-positive Kupffer cells (Fig. 6D, red arrows; and 6E, yellow arrows) but also in hepatocytes (Fig. 6E, green arrows) in the liver of the MPS II at 10 months post-CBSCT. These results indicate that there were many IDS-positive cells were widely distributed not only among Kupffer cells but also among hepatocytes in the liver at 10 months after CBSCT.

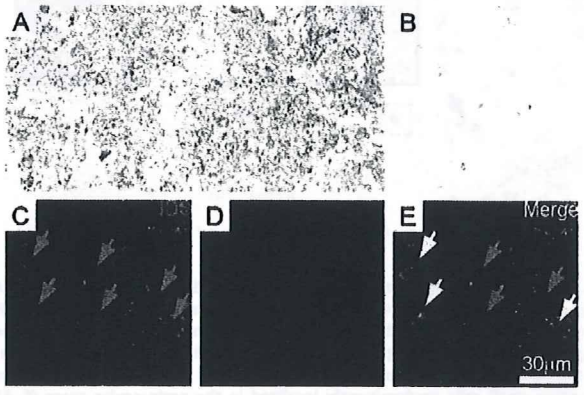

Fig. 6. Immunohistochemical studies on IDS in the liver of MPS II post-CBSCT. (A and B) IDS-immunoreactivity is extensively detected in many cells (A); however, it disappears completely when the antibody is preabsorbed with the excess recombinant human IDS (B). (C–E) Double immunofluorescence for IDS and CD68. IDS-immunoreactivity is detected in many cells (C, green arrows), not only in CD68-positive Kupffer cells (D, red arrows; and E, yellow arrows) but also hepatocytes (E, green arrows). The blue fluorescence is from DAPI.

3.4.2. Distribution of IDS-positive cells in the CNS

IDS-immunoreactivity of the antibody was more evident in the formalin-fixed frozen sections of the non-MPSII control (Fig. 7A) than in the unfixed-frozen sections (Fig. 5B), but the antibody was not reactive with the paraffin sections (data not shown). Therefore, we used the formalin-fixed frozen CNS samples in the following studies.

In the immunohistochemical study on IDS of the non-MPS II control and MPS II post-CBSCT, many IDS-immunoreactive large cells, presumably neurons, were found in the non-MPS II control (Fig. 7A and B); whereas only a few IDS-immunoreactive small cells, reminiscent of microglia/monocytes, were found in the MPS II post-CBSCT (Fig. 7C–F). Most IDS-positive small cells were localized in perivascular spaces in the MPS II post-CBSCT (Fig. 7C–E). Although many of them seemed to be in the distended Virchow-Robin spaces (Fig. 7C, area between red dashed lines), some of them were evidently found in the brain parenchyma (Fig. 7E, arrows). Also, a very small minority of them were present in the parenchyma where no blood vessels were found in the neighborhood (Fig. 7F). On the other hand, no IDS-immunoreactive neurons were found in the MPS II post-CBSCT.

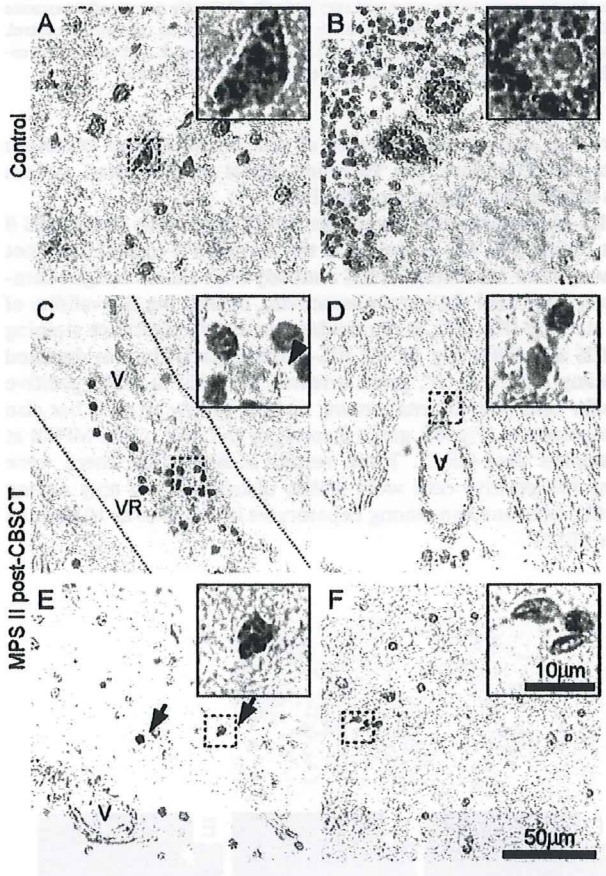

Fig. 7. Immunohistochemical studies on IDS in the CNS of non-MPS II control (A and B) and MPS II post-CBSCT (C–F). A, C, and E, cerebrum; B and D, cerebellum; F, hippocampus. Insets represent higher magnification of the rectangle outlined in the corresponding figures, focusing on IDS-positive cells. The non-MPS II control has many IDS-positive neurons (A and B), whereas the MPS II post-CBSCT has only a few IDS-positive small cells predominantly localized in the perivascular space (C–E). Many of IDS-positive cells in the MPS II post-CBSCT seemed to be in the distended Virchow–Robin space (C, area between red dashed lines); however, some of them have migrated into the brain parenchyma nearby the blood vessel (E, arrows). A very small number of them are present in the parenchyma where no blood vessels are found in the neighborhood (F). V, blood vessel; VR, Virchow–Robin space, and arrowhead: an enlarged cell with many vacuoles in the Virchow–Robin space (see also Fig. 3C).

We next performed double immunofluorescence staining for IDS and Lamp2, NSE, transferrin, S-100 or CD68. IDS-immunoreactivity in the cerebrum of the non-MPS II control was observed in NSE-positive neurons (Fig. 8A–C) and transferrin-positive oligodendrocytes (Fig. 8D–F) but not in S-100-positive astrocytes (Fig. 8G–I). Although CD68-positive microglia/monocytes were only few in number, they were also immunoreactive for IDS (Fig. 8J–L). On the other hand, IDS-immunoreactivity in the cerebrum of the MPS II post-CBSCT was co-localized with that of Lamp2 (Fig. 8M–O) and found exclusively in CD68-positive cells (Fig. 8P–R) but neither in NSE-, transferrin- nor S-100-positive cells (data not shown). A cell count showed that about 5.7% (17/297) of CD68-positive microglia/monocytes had IDS-immunoreactivity.

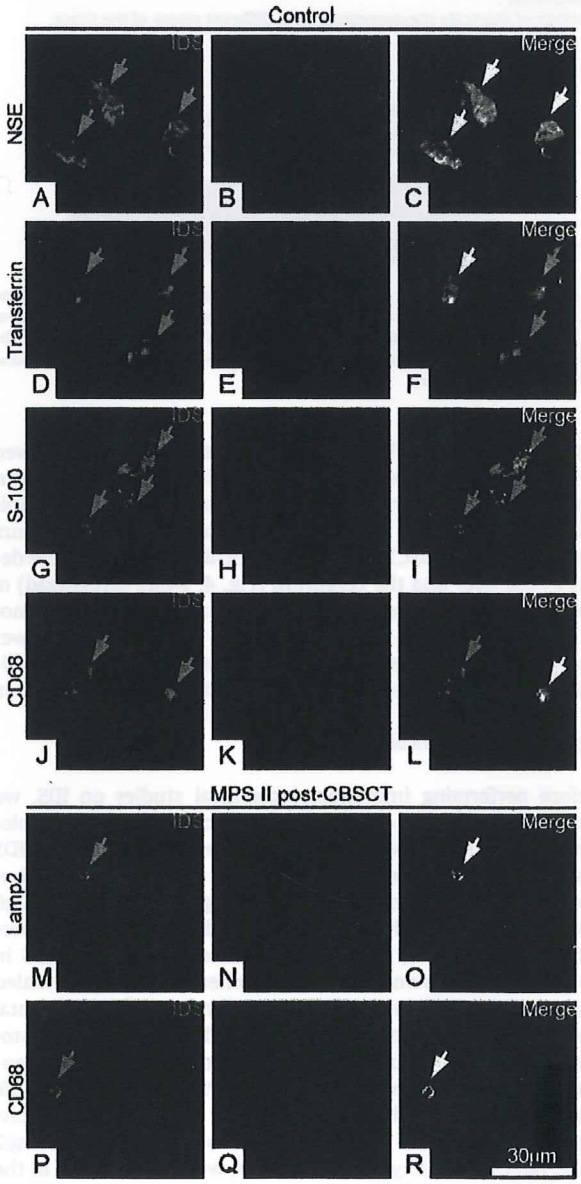

Fig. 8. Double immunofluorescence in the cerebrum for IDS and NSE (A–C), transferrin (D–F), S-100 (G–I) or CD68 (J–L) of the non-MPS II control; and for IDS and Lamp2 (M–O) or CD68 (P–R) of MPS II post-CBSCT. Green arrows indicate IDS-positive cells; and red ones, NSE-, transferrin-, S-100-, CD68- or Lamp2-positive cells. Yellow ones indicate double-positive cells. The blue fluorescence is from DAPI. IDS-immunoreactivity in the non-MPS II control is observed in NSE-, transferrin-, and CD68-positive cells but not in S-100-positive cells. On the other hand, IDS-immunoreactivity in the MPS II post-CBSCT is co-localized with that of Lamp2, and observed exclusively in CD68-positive cells.

4. Discussion

In this study, we evaluated the CNS pathology of a 6-year-old MPS II male who died at 10 months post-CBSCT. There were many distended cells with accumulated substrate in the CNS, like in untreated cases previously reported [3–5]; and IDS enzyme activity there remained very low. However, IDS-immunoreactivity was found in a few microglia/monocytes predominantly localized in the perivascular spaces. Furthermore, as far as we know, this study also identified the IDS-positive cells in the normal brain for the first time. The fact that oligodendrocytes expressed IDS in the non-MPS II control, but not in the MPS II patient, may partly be the reason for the white matter lesions seen in MPS II [4,29], since impairment of oligodendrocytes due to IDS deficiency may lead to the secondary dysmyelination.

The antibody we used was produced in goats immunized with recombinant human IDS (2449-SU, R&D Systems Inc.), which contains whole mature protein (Ser 26–Pro 550); therefore, there might be some residual recipient IDS detected by the antibody *in vitro*. However, this was not the case *in situ* because nonsense-mediated mRNA decay generally degrades mRNAs that terminate translation more than 50–55 nucleotides upstream of a splicing-generated exon–exon junction such as in our patient [30]. In addition, Chang et al. reported in an expression study *in vitro* of the W267X mutation of the IDS gene that the truncated IDS proteins produced seemed to be trapped in the endoplasmic reticulum, not in the lysosomes, of transfected COS-7 [31]; therefore, the trapped proteins were probably degraded by proteasomes [32]. Furthermore, even if there were the residual recipient IDS proteins despite of the conditions described above, it is very unlikely that they would exist exclusively in microglia/monocytes predominantly localized in the perivascular spaces. These lines of evidence indicate that the IDS-immunoreactive cells in our MPS II post-CBSCT patient are judged to be donor-derived microglia/monocytes that had penetrated the blood vessels.

The precise physiological events after HSCT still remain to be determined. Especially, the efficiency of microglia replenishment by hematopoietic stem cells has remained controversial [33]. In a study on B6/129 F2 mice after bone marrow transplantation (BMT), Kennedy and Abkowitz found that donor microglia represented only 30% of the total microglia at 12 months and that the donor cells were predominantly seen at perivascular and leptomeningeal, but not parenchymal, sites; although 89% of the splenic monocytes/macrophages were of donor origin by 1 month [34]. Furthermore, in the same report they also showed that the engraftment rate of Kupffer cells in the liver was higher than that of microglia in the CNS at 6 months after BMT (36% vs. 23%) and that the engraftment rate increased at 12 months after BMT (52% vs. 30%). Cogle et al. demonstrated that transgender microglia, containing a Y chromosome, made up 1–2% of all microglia and that transgender neurons and astrocytes were present in the hippocampus of female patients up to 6 years after HSCT [18]. On the other hand, microglia progenitor recruitment from the circulation was not found in denervation or CNS neurodegenerative disease of chimeric animals obtained by parabiosis without brain conditioning procedures such as irradiation [35]. The discrepancy about the microglia progenitor recruitment in the CNS might reflect the difference in neurological disorders, the age, and the conditioning regimen for HSCT. In our study, IDS-immunoreactivity was found in about 5.7% of the microglia/monocytes predominantly localized in perivascular spaces of the patient at 10 months post-CBSCT. Our result is largely compatible with the above-mentioned study by Kennedy et al. The engraftment rate might have been higher if the patient had lived longer. On the other hand, we detected no IDS-immunoreactivity in neurons, oligodendrocytes or astrocytes of the patient.

HSCT has been reported to be much more effective in the liver than in the CNS [16,34,36]. Resnick et al. found that liver biopsy specimens from MPS patients who had achieved metabolic correction by BMT were not stainable with colloidal iron [37], although those from untreated cases had hepatocellular dilatation with rarefaction of the cytoplasm, which gave positive staining with colloidal iron [38]. In addition, in a study performing BMT on model mice of Hurler disease (MPS I: MIM +607014), Zheng et al. reported the disappearance of the storage vacuoles in both Kupffer cells and hepatocytes, and the broad distribution of α-L-iduronidase (EC 3.2.1.76), a deficient enzyme of MPS I, with sparse distribution of the bone-marrow derived cells in the liver [14]. In our case, the hepatomegaly was clinically improved at 7 months post-CBSCT, and hepatocytes and Kupffer cells did not appear to be swollen with apparent intracytoplasmic colloidal iron-positive substrate at 10 months post-CBSCT. Also, a higher intensity of the donor-derived band was detected in the liver than in the cerebrum by VNTR analysis. Furthermore, the liver showed about 40% of the normal IDS enzyme activity, and many IDS-immunoreactive Kupffer cells and hepatocytes. Although evaluation of the hepatic pathology of the patient before CBSCT had been not performed, it is very unlikely that residual IDS activity was present in a tissue-specific way such that the residual IDS activity was preserved only in the liver. Therefore, IDS-immunoreactivity in the liver after CBSCT was judged to be donor-derived. Our result and those of previous studies suggest that donor-derived migrating cells, such as Kupffer cells, secreted IDS enzyme that was taken up by neighboring recipient hepatocytes and Kupffer cells sufficiently to correct GAG metabolism in the liver of the MPS II patient at 10 months post-CBSCT.

There are some studies documenting that accumulated substrate persists in the peripheral nerves of MPS II patients for up to 2 years after HSCT [36,39]. With regard to other inherited metabolic diseases, Will et al. reported that pronounced cytoplasmic vacuolation was seen in the brain of a 7-year-old boy with α-mannosidosis (MIM #248500) at 18 weeks post-BMT [16]. Although the precise evaluation of peripheral nerves was impossible, our data seem to be compatible with their study showing that the accumulated substrate in neural tissues had not diminished less than 2 years after HSCT.

There are many clinical studies and reviews that have concluded that HSCT does not significantly alter the natural history of severe cases of MPS II [40–44]. Guffon et al. reported that 4 MPS II children, whose development or intelligence quotient at BMT had been from 65 to 70, had deteriorated after BMT [44]. On the other hand, in a study of 54 MPS I children, many patients could achieve a favorable long-term outcome after successful BMT [45]. The reason for the neurologically poorer outcome in MPS II than in MPS I is not clear; however, there may be two explanations for the different efficacy. First of all, it may be because the diagnosis and treatment are generally delayed more in children with MPS II due to the slower onset and progression than in those with MPS I. Indeed, Escolar et al. concluded that CBSCT in newborns with infantile Krabbe's disease (MIM #245200), but not symptomatic babies, favorably altered the natural history of the disease. Secondly, it may be because HSCT itself may be less effective in the CNS of MPS II than in that of MPS I even at the early stage of disease. Zheng et al. reported a reduction in the number of vacuolated neurons and migration of hematopoietic donor cells to the brain of MPS I mice with retrovirally transduced bone marrow [14], and Ellinwood et al. reported a significant decrease in the brain GAG levels of MPS I felines after BMT [13]. In addition, Bikenmeier et al. reported a dramatic reduction in storage of accumulated substrate in perivascular and meningeal cells, but not in neurons and scattered glial cells, in the brain of MPS VII mice after BMT [46]. In our study, there were many dis-

tended cells with accumulated substrate and only a few IDS-positive microglia/monocytes in the CNS of our patient. The absence of IDS-immunoreactivity in oligodendrocytes or neurons can be due to either the true absence of IDS in these cells (the absence of effective transfer of enzyme) or the limitation of the immunohistochemical method to detect IDS present at low concentrations in these cells. Our findings might explain the reason why HSCT fails to arrest neurological progression in MPS II. However, despite our results and those of the previous studies described above, evidence for the reason for this issue has been insufficient yet; because there have been no studies about the brain pathology of any type of MPS patients, not model animals, after HSCT. Therefore, the issue may need more neuropathological studies on human MPS patients after HSCT.

Our study is unique and valuable in evaluating the efficacy of HSCT by detecting the donor-derived cells; since there has been only one report documenting the fate of donor-derived cells after HCST in patients with inherited metabolic diseases. Schonberger et al. reported that immunohistochemical staining for adrenoleukodystrophy (ALD, MIM #300100) protein revealed no differences between the brain of a 15-year-old ALD patient at 76 days post-HSCT and that of the control [17]. Their results are in sharp contrast with the study by Yamada et al., who documented that little ALD protein was detected in the brain of an ALD model mouse at 6 months post-HSCT [47]. After evaluation of the accumulated substrate and IDS enzyme activity, we conclude that only a few donor-derived cells had penetrated into the CNS of our MPS II patient at 10 months post-HSCT and that the number of donor-derived cells was insufficient for metabolic improvement. However, our findings showing the existence of donor cells in the brain parenchyma at 10 months post-CBSCT suggest the potential of HSCT for treatment of MPS II.

The issue of HSCT for LSD patients requires detailed discussion regarding the indication, timing, and conditioning regimen. Therefore, the accumulation of neuropathological evidence, such as our study, is very valuable for establishment of evidence-based protocols of HSCT for these patients.

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We obtained unfixed-frozen cerebrum samples from an 11-year-old untreated MPS II patient and a 12-year-old non-MPS II control from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA. The role of the NICHD Brain and Tissue Bank is to distribute tissue; and, therefore, it cannot endorse the studies performed or the interpretations of results.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymgme.2009.05.006.

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REVIEW ARTICLE

Pediatric post-transplant lymphoproliferative disorder after cardiac transplantation

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Abstract Post-transplant lymphoproliferative disorder (PTLD) is a well recognized and potentially fatal complication after pediatric cardiac transplantation. PTLD encompasses a wide spectrum, ranging from benign hyperplasia to more aggressive lymphoma. Most cases are Epstein-Barr virus (EBV)-related B-cell tumors resulting from impaired immunity due to immunosuppressive therapy. Pediatric recipients, often seronegative for EBV at transplantation, have a greater risk for PTLD than adults. The clinical presentation of PTLD varies from isolated lymphadenopathy to systemic disease; common sites involved are gastrointestinal tract, lung or airway, and cervical lesions. Timely and accurate diagnosis based on histological examination of biopsy tissue is essential for early intervention. Immunostaining for EBV and evaluation for clonality are needed. For prophylaxis when EBV viral loads are increasing or for initial treatment of early lesions or polymorphic PTLD, a reduction in immunosuppressive treatment is a key component of therapy, but caution is needed for possible rebound allograft rejection. Chemotherapy is indicated for patients with poor response to reduced immunosuppression and for highly aggressive monomorphic PTLD. The use of rituximab in combination with chemotherapy is effective. For the time being, avoiding excessive immunosuppression is the most effective strategy for reducing the incidence of PTLD. Calcineurin inhibitor (CNI) minimization with proliferation signal inhibitors (PSIs) or conversion from a CNI to a PSI might be useful for preventing both development of PTLD and allograft rejection.

Keywords Cardiac transplantation · EBV · Immunosuppression · Pediatric · Post-transplant lymphoproliferative disorder

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1 Introduction

The number of pediatric heart transplant procedures, including re-transplants, has been estimated to be around 300–350 annually worldwide [1]. Despite the excellent long-term survival currently achieved in this population, significant morbidity and mortality remain as a consequence of the immunosuppressive medications required to prevent allograft rejection. Malignancy is one such complication; lymphoma is most common. Lymphoma is counted as the cause of death in 2.0, 4.5, 2.9, and 8.5% of cases in the period from 31 days to 1 year, >1–3 years, >3–5 years, and >5 years after transplantation, respectively, according to the report from the International Society for Heart and Lung Transplantation [1]. Post-transplant lymphoproliferative disorder (PTLD) encompasses a wide spectrum of disease manifestations, ranging from a benign plasmacytic hyperplasia and infectious mononucleosis-like PTLD to an aggressive monoclonal lymphoma that can be fatal. Pathogenesis, incidence, risk factors, pathology, clinical presentation, diagnosis, treatment, and prophylaxis are discussed.

2 Pathogenesis: the role of Epstein-Barr virus

Epstein-Barr virus (EBV) infection, in the setting of immunosuppression, plays a central role in the

pathogenesis of PTLD. Naive EBV-infected B cells display the growth latency program (type III latency), in which EBNA1-6, LMP1, LMP2A, and LMP2B are expressed, resulting in polyclonal cell proliferation [2–4]. The local microenvironment (cytokine milieu) may lead to an emergence of specific clones, resulting in oligoclonal or monoclonal proliferation. Normally, to escape elimination by EBV-specific cytotoxic T lymphocytes (CTLs), these EBV-infected cells switch to the default program (type II latency) in which EBNA2 is silenced, and EBNA, LMP1, and LMP2A are expressed, and differentiate into memory cells [3]. Latent infection results in permanent lodging of EBV in a more restricted latency program (type 0 latency) with expression of EBER and LMP2A only [3]. From there, in immunosuppressed patients, EBV reactivation may lead to persistent lytic infection with high viral load in peripheral blood lymphocytes. B cells infected with reactivated EBV can seed the peripheral lymphoid system, proliferate, and progress to PTLD with type III latency [5]. Whereas, type I latency, defined by expression of EBER and EBNA1, has been documented only in Burkitt lymphoma [3].

In type III latent cells in immunosuppressed transplant recipients, rates of proliferation may exceed rates of clearance and differentiation, leading to plasmacytic hyperplasia and polymorphic PTLD. In the continued presence of immunosuppression, some of the cells acquire additional cytogenetic abnormalities such as BCL6 mutations [6], c-myc rearrangement, or disruption of p53 tumor suppressor genes, resulting in a more malignant form, monomorphic PTLD [7, 8].

EBV is associated with pediatric PTLD after cardiac transplantation in 83–87% of patients [9, 10], while primary EBV infection is present in 50–68% [9, 10]. Patients experiencing primary infection are at higher risk because of delays in the development of the CTL response and the absence of neutralizing antibodies. In solid organ transplant patients, either in case of primary infection or reactivation of EBV, the PTLD cells are typically of recipient origin, while, in hematopoietic stem cell transplantation (HSCT) recipients, most cases of PTLD are typically donor origin because the host lymphoid cells are eradicated by conditioning and eventually replaced by donor lymphocytes [11, 12].

The vast majority of PTLD cases are B-cell-associated, while T-cell PTLD is rarely seen [13, 14]. The Pediatric Heart Transplant Study (PHTS) reported a single case (2%) with T-cell PTLD among 48 cases after cardiac transplantation [9]. Pediatric T-cell PTLD after solid organ transplantation was associated with EBV in 21–43% but not in the remainder [14, 15].

It is important to note that not all cases are associated with EBV. EBV-negative PTLD often occurs more than 3–5 years after transplantation; the majority of these cases are B-cell PTLD, although a small proportion (27%) is T-cell PTLD [16, 17]. The clinical outcome in patients with EBV-negative PTLD is poor with a median survival time of 1–7 months [16, 17]. Given the relatively rarity of these EBV-negative PTLDs, their pathogenesis is still poorly understood.

3 Incidence

The incidence of PTLD after solid organ transplantation is markedly different in children as compared to adults and also varies according to the type of organ transplant because of differences in the intensity of immunosuppressive regimens used [18, 19]. In adults, kidney recipients have the lowest frequency of PTLD (1–2%); liver recipients have a slightly higher incidence (1–3%), followed by adult heart recipients (1–6%), heart-lung recipients (2–6%), and lung recipients (4–10%); and small bowel recipients have the highest incidence (up to 20%) [19]. Children have a much higher incidence of PTLD than adults: 1–10% in kidney transplants; 4–15% in liver transplants, and 6–20% in lung, heart, and heart-lung transplants [19].

The PHTS Group reported an overall 5% incidence for pediatric PTLD among 1,184 patients after cardiac transplantation [9]. Freedom from PTLD was 98% at 1 year and 92% at 5 years after transplantation. An early phase of peak risk was recorded at around 6 months, followed by a continuous slow decrease in risk, but without ever reaching zero. In contrast, in HSCT recipients, the onset of PTLD is earlier: within 6 months (median 70–90 days) after HSCT [12]. A more long-term follow-up study of pediatric cardiac transplant patients from Stanford University reported a high incidence of PTLD in five of 52 patients (9.6%) who survived longer than 10 years after transplantation [20]. The same group also showed a higher incidence of PTLD in children (11%) than in adults (3.4%) among more than 1,000 heart and heart-lung recipients [21]. The higher incidence of PTLD in pediatric recipients is mainly attributed to the development of primary EBV infection after transplantation, as described earlier.

4 Risk factors

Recipient age is a risk factor for PTLD, as previously described. A higher frequency of transplant rejection is another risk factor for PTLD [21]. This may be related to the increased immunosuppression needed to treat allograft rejection.

Primary EBV infection after cardiac transplantation is also a risk factor, as mentioned earlier; therefore, EBV

status mismatch between recipient and donor (seronegative recipient with seropositive donor) is associated with the development of PTLD. A German Group has reported that pre-transplant EBV-seronegative status was significantly ($p = 0.001$) more frequent in patients with PTLD (6 of 12, 50%) as compared to those without PTLD (11 of 50, 18%) [10]. In HSCT recipients, EBV-seronegative donor cells can be infected with EBV after HSCT; primary infection of donor cells, in either EBV-seropositve or -seronegative recipients, is also risk factor for the development of PTLD [12].

B-cell PTLD occurring early after transplantation is often associated with high EBV viral loads in peripheral blood samples and these high viral loads often precede clinical symptoms. Green et al. [22] used a pre-emptive protocol based on EBV PCR threshold surveillance, which led to a decrease of PTLD incidence from 46 to 16% in pediatric intestine recipients. They used a threshold of $\geq$200 genome copies/10^5 lymphocytes in EBV-seropositive children and $\geq$40 genome copies/10^5 lymphocytes in pre-transplant EBV-seronegative children. Other reports used a cutoff for elevation of >3000–4000 copies/μg DNA [23, 24]. Allen et al. [25] reported a higher mean baseline EBV load in pediatric recipients with PTLD as compared to controls (3.1 $\log_{10}$ vs. 1.6 $\log_{10}/10^6$ PBMCs), with every 1 log increase in viral load resulting in a threefold increase in the likelihood of PTLD. A chronic high viral load state with the presence >16,000 genome copies/mL whole blood was reported to be a predictor of de novo or recurrent PTLD after pediatric heart transplantation [26]. Thus, EBV serostatus and monitoring EBV viral load is essential for suspecting PTLD and its early management.

In general, high viral loads have been found to be sensitive but not specific predictors of PTLD development in surveillance studies involving pediatric recipients. Elevated EBV viral loads without clinical symptoms do not seem to be specific for the development of PTLD; EBV viral loads alone cannot be used to identify individual patients who develop PTLD [27]. It must also be noted that EBV-associated PTLD has been described in patients with low or undetectable EBV viral loads [28].

PTLD arises, to a large part, as a consequence of immunosuppression necessary to prevent allograft rejection. An increased risk for PTLD has been reported to be associated with the use of antithymocyte globulin or OKT3 [18, 29, 30]. Reports, primarily in pediatric liver transplantation, have shown a two to fivefold increase in the risk of PTLD developing in patients treated with tacrolimus as the primary immunosuppressant drug as compared to cyclosporine [31–33]. The total incidence density rate of PTLD was 4.86 and 0.49 per 100 patient-years in the primary tacrolimus- and cyclosporine-treated patients, respectively [33]. A recent study showed that no such significant association was observed with the use of tacrolimus for PTLD in a more recent era (1996–2000), presumably because of the lower target plasma tacrolimus concentration (5–10 ng/mL) [34]. The use of mycophenolate mofetil, a more recently introduced immunosuppressive agent, has not been associated with an increased risk of PTLD development [34–36]. The effect of the proliferation signal inhibitors (PSIs), sirolimus and everolimus, on PTLD development is not yet clear [35, 36]. These drugs might be associated with reduced PTLD development because they display an inhibitory effect on PTLD-derived cells in vitro and in vivo in an animal model [37, 38].

HLA class I tetramers folded with EBV peptides are used to detect EBV-specific CTLs [39–41]. Pediatric transplant recipients, even receiving immunosuppression, have been reported to be able to generate EBV-specific CTLs [39]. Tetramer analysis may be useful in monitoring EBV infection in post-transplant patients [39].

A disparity of HLA mismatch has also been reported to be associated with PTLD development. Increased total numbers of HLA mismatches were found to be associated with PTLD [35]. Mismatches at HLA-B loci were reported to contribute to greater risk [42]. Another study showed the association of PTLD with the expression of HLA-B18, HLA-B21, or HLA-DR7 [43]. Decreased surveillance by T cells with dual specificity for EBV as well as for alloantigens on the allograft might facilitate clonal expansion of B cells latently infected with EBV [44].

5 Pathology

PTLD can be divided into three distinct morphological groups, as reported by the World Health Organization classification of tumors of hematopoietic and lymphoid tissues (Table 1) [45]. The first group comprises diffuse B-cell hyperplasia, characterized by architectural preservation of the involved lymphoid tissue. Plasmacytic hyperplasia and infectious mononucleosis-like PTLD are often seen in children and young adults. The early lesions involve lymph nodes or tonsils and adenoids more often than true extranodal sites [46]. They may respond well to a reduction in immunosuppression [47–49]. The second group comprises polymorphic PTLD (Fig. 1a), polyclonal or more commonly monoclonal [19], which is composed of immunoblasts, plasma cells, and lymphoid cells that efface the architecture of lymph nodes or form destructive extranodal masses. In pediatric heart transplantation, this is the most common type of PTLD and frequently follows primary EBV infection [9]. The third group comprises monomorphic PTLD, exclusively monoclonal, more aggressive B-cell or T/NK-cell neoplasms. Monomorphic

Table 1 World Health Organization classification of post-transplant lymphoproliferative disorders [45]

Early lesions
Plasmacytic hyperplasia
Infectious mononucleosis-like lesion
Polymorphic PTLD
Monomorphic PTLD
B-cell neoplasms
Diffuse large B-cell lymphoma
Burkitt lymphoma
Plasma cell myeloma
Plasmacytoma-like lesion
Other
T-cell neoplasms
Peripheral T-cell lymphoma, NOS
Hepatosplenic T-cell lymphoma
Other
Classic Hodgkin lymphoma-type PTLD

NOS not otherwise specified, *PTLD* post-transplant lymphoproliferative disorder

B-cell PTLD can be further divided into diffuse large B-cell lymphoma (DLBL) (Fig. 1b, c) and, less often, Burkitt lymphoma or plasma cell myeloma. Monomorphic T-cell PTLD can be divided according to whether it is large cell, anaplastic, or non-specific. Classic Hodgkin lymphoma can occur rarely in the post-transplant setting [50, 51], although the distinction of Hodgkin-like PTLD from true Hodgkin lymphoma is difficult [52].

The relation between histology and timing of onset has been recorded for the PHTS cohort [9]. Excluding early lesions, 71% of early-onset cases with PTLD developing less than 3 years from transplantation were polymorphic, while 57% of the late-onset cases developing more than 3 years from transplantation were monomorphic.

6 Clinical presentation

The clinical presentation of PTLD ranges from an isolated abnormal lymphadenopathy to fulminant systemic disease. Most patients present with lymphadenopathy, fever, or non-specific symptoms such as tonsillitis and weight loss. It can also affect non-lymphoid organs, including the allograft itself. When beginning in the tonsillar tissue and Waldeyer ring, noisy breathing, new onset of snoring, or changes in voice can be the only symptoms. Rapid growth of the tumor can lead to upper airway obstruction. Such otolaryngologic presentation, reported to be 60–86% in some studies of pediatric transplant recipients [53, 54], may have the early non-destructive form of PTLD with massive plasmacytic hyperplasia.

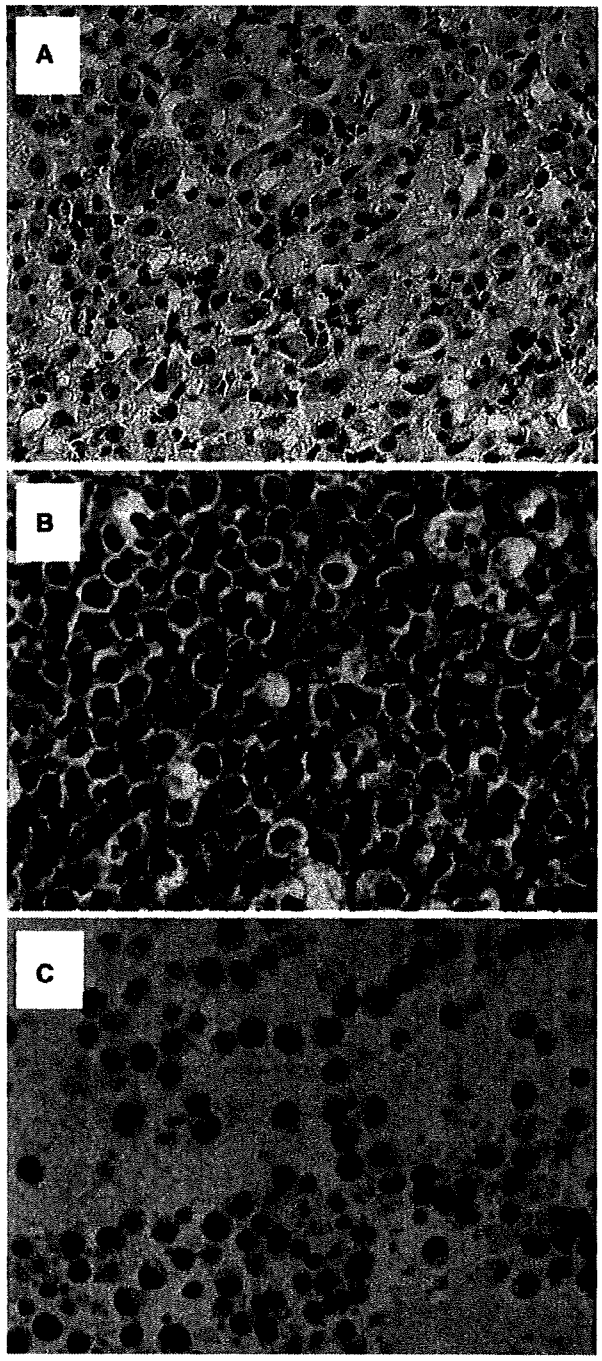

Fig. 1 Pathology photomicrographs of pediatric post-transplant lymphoproliferative disorders (PTLDs): polymorphic B-cell PTLD in a 5-year-old girl (**a**) and diffuse large B-cell lymphoma in a 3-year-old boy (**b, c**). **a** Infiltration of large lymphoid cells ($CD20^+$, data not shown) mixed with small lymphoid cells ($CD3^+$, data not shown) and histiocytes, displaying polymorphic appearance (hematoxylin and eosin stain, ×400). **b** Diffuse infiltration of large lymphoid cells ($CD20^+$, data not shown) with clear nucleoli (hematoxylin and eosin stain, ×400). **c** Tumor cells showing reactivity stained brown with a probe specific for EBER-RNA (EBER1 and 2 in situ hybridization, ×400); **b** and **c** are reproduced with permission from Kusuki et al. [98] by the courtesy of International Journal of Hematology

In the PHTS cohort, in which early non-destructive lesions were excluded, roughly equal numbers of patients had a single site of disease compared with multiple sites [9]. The most common sites were: gastrointestinal tract (39%); lung or airways (25%); and cervical adenopathy (18%). Central nervous system disease occurred in only 3.6%. Gastrointestinal symptoms at presentation included diarrhea, vomiting, anorexia, abdominal pain, and lower gastrointestinal bleeding. Tai et al. [55] reported an increased risk of mortality in pediatric PTLD patients with abdominal involvement. In this series, eight patients had abdominal involvement; four required surgical interventions because of intussusception and bowel perforation ($n = 1$), bowel perforation ($n = 2$), or tumor debulking ($n = 1$). Four of the eight patients died from complications related to PTLD.

The most fulminant form presents like acute infectious mononucleosis and progresses to a clinical picture resembling septic shock, with tumor involvement of all organs, multisystem organ failure, and disseminated intravascular coagulation [56, 57]. Sometimes, this presentation coincides with a rejection episode. Caution is needed in the differential diagnosis of this form of PTLD as treatment with added immunosuppression can prove fatal.

Primary EBV infection is a risk factor for PTLD, as described earlier, and possibly causes more severe disease than EBV reactivation. At least in the setting of cord blood transplantation, EBV reinfection (secondary primary infection) displays more aggressive disease than EBV reactivation [58].

Bone marrow involvement may present with new onset or persistent cytopenia. Hemophagocytosis is occasionally observed in EBV-related T-cell PTLD [59–61], but is rare in B-cell PTLD [60, 62]. Polymorphic PTLD may present with features overlapping early lesions and monomorphic PTLD. As a result of the variability of presentation, clinicians should have a suspicion of PTLD in any patient with a history of transplantation.

7 Diagnosis

The timely and accurate diagnosis of PTLD is essential for early intervention. Monitoring of EBV viral loads can be helpful for suspecting or predicting the development of PTLD in most cases, though not all cases. The diagnosis of PTLD should be based on histological examination of biopsy tissue [63]. Excision biopsy is preferable; needle aspiration has little role. In situ hybridization with the EBER-1 probe [64], which labels EBV-encoded RNA in infected cells, is the most reliable histologic stain for the presence of EBV (Fig. 1c) and its use is recommended in all cases of suspected EBV disease [63]. Analysis of EBV latent proteins such as EBNA2 and LMP1 is useful for identifying the type of latency. EBNA2 and LMP1 viral proteins are expressed in type III latent immunoblasts of monomorphic PTLD with the exception of PTLD with plasmacytic differentiation [65]. Meanwhile, EBV-infected lymphocytes in early and polymorphic PTLDs represent a mixture of latencies II, III, and in at least one-third of infected cells, latency 0 [3].

Other useful tests include flow cytometric analysis of immunophenotype to determine the type of cellular infiltrates and genetic evaluation for tumor clonality. Clonality can be assessed using immunoglobulin heavy chain gene rearrangement and EBV termini assays [66]. Flow cytometric analysis can also be used to determine monoclonality of B-cell lesions and T-cell lesions when immunoglobulin light chain (κ or λ) restriction or loss of surface or cytoplasmic immunoglobulin and abnormal T-cell antigen expression or loss is identified, respectively [67]. Association between clonality and prognosis is controversial. A study of pediatric PTLD after solid organ transplantation reported that monoclonality did not imply poor prognosis [68].

Staging is assessed using the same system as non-Hodgkin lymphoma in the normal population [69]. Staging of the disease should include computed tomography (CT) of the abdomen and thorax, and bone marrow aspiration study. Fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET)/CT scan has been reported to be useful for not only diagnosis of PTLD but also evaluation of response to treatment [70, 71]. It must be noted that PET cannot distinguish between tumor and chronic inflammation. The presence of PTLD within the graft may sometimes be mistaken for acute rejection; immunostaining for EBV and evaluation for clonality may be helpful for differential diagnosis [72]. Pertinent blood tests include complete blood counts, serum lactate dehydrogenase levels, blood levels of EBV viral loads, and serum levels of EBV antibodies.

8 Treatment

The treatment strategy for PLTD varies according to the histology of the disease itself (Table 2). Initial treatment in all patients with PTLD is to reduce immunosuppression in the hope that this will restore CTL function to increase anti-tumor activity. Anti-metabolite and purine inhibitor drugs are stopped and any calcineurin inhibitor (CNI), tacrolimus or cyclosporine, is typically decreased over 4–6 weeks to a low level (around 25–25% of the normal therapeutic level) depending on the extent of disease, while the patient is watched closely for regression and also, equally importantly, for rejection. A response to reduction in immunosuppression is usually seen within 2–4 weeks [63]. Reduction in immunosuppression alone leads to

Table 2 Potential management strategy of PTLD

Increasing EBV viral loads or early lesion
Reduce immunosuppression + antivirals
Polymorphic PTLD
Reduce immunosuppression
Rituximab
For persistant disease, the same treatment for monomophic PTLD
Monomorphic PTLD
Chemotherapy ± rituximab
For all patients, monitor for rejection
Consider PSIs for prevention of rejection

PTLD post-transplant lymphoproliferative disorder, *EBV* Epstein-Barr virus, *PSIs* proliferation signal inhibitors

long-term disease remission in 40–86% of cases of PTLD in pediatric transplant recipients [47–49]. This treatment can succeed in early lesion or polymorphic PTLD but is less successful in monomorphic types. Minimization of immunosuppression, however, often causes allograft rejection, requiring careful observation on allograft function (see discussion as follows).

Since PTLD is usually due to neoplastic proliferation of B cells, the use of monoclonal antibodies against CD20 is a logical therapeutic approach. Rituximab is a chimeric mouse/human monoclonal antibody that binds to the CD20 antigen on the surface of most normal and malignant B cells, and can induce lysis of antigen-positive cells. In one series of pediatric transplant recipients with PTLD, 11 of 13 children had complete resolution of the disease after administration of 1–4 doses of rituximab (375 mg/m^2 per dose) alone [73]. Other studies have shown remission rates of 44–87% with the use of rituximab, some with concomitant reduction in immunosuppression [74–76]. In addition, rituximab has been widely used for treatment of B-cell lymphoma as an adjunct to chemotherapy in non-transplant populations and reported to be effective compared to treatment with standard chemotherapy alone [77–80]. Rituximab may allow lowering of chemotherapy doses in attempt to reduce toxicity, particularly in pediatric patients [73]. In a study of six pediatric transplant recipients with PTLD who were treated with rituximab and reduced dose chemotherapy, five showed a complete response with only limited toxicity [81]. Long-term follow-up studies are needed for full evaluation of the use of rituximab because it does not restore the CTL activity essential for long-term EBV control and relapse may therefore be a problem.

Chemotherapy, often combined with rituximab, is commonly used in the treatment for patients with PTLD who do not respond to reduction in immunosuppresion. It is also used in combination with reduced immunosuppression as initial therapy for more aggressive monomorphic PTLD, often late-onset PTLD. The CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) regimen has been most frequently used. A large retrospective study by Elstrom et al. [82] reported the efficacy of CHOP chemotherapy with or without rituximab. In this study, 23 patients received chemotherapy (CHOP, $n = 10$; rituximab plus CHOP [R-CHOP], $n = 9$; other regimens, $n = 4$) with an overall response rate of 74%. Currently, rituximab plus combination chemotherapy, such as R-CHOP, may be a more suitable option for the treatment of PTLD because of concurrently maintaining immunosuppression for allograft protection. However, caution is needed regarding the toxicity of combination chemotherapy [82]. Low-dose chemotherapy, consisting of cyclophosphamide and prednisone, has been used in pediatric transplant recipients with 67% failure-free survival (without PTLD and with functioning original allograft) after failure with front-line therapy [56]. It may be effective by simultaneously controlling PTLD, preventing or treating allograft rejection, and minimizing treatment-related mortality.

The PHTS study showed lower rejection rates when chemotherapy was used as primary therapy [9]. In the subgroup with monomorphic disease, patients receiving chemotherapy as first-line therapy had slightly better 2-year survival (75%) than those receiving reduced immunosuppression (57%). Of the patients with polymorphic PTLD treated with reduced immunosuppression as first-line therapy, 61% developed rebound acute cellular rejection during the first 6 months after diagnosis of PTLD. A fine balance between management of PTLD and preserving allograft from rejection is therefore highly important.

After complete remission of PTLD is achieved, the patient still requires immunosuppression to protect the allograft. Immunosuppressive drug dosage should be maintained within the levels effective to prevent both rejection and relapse of the PTLD. The PHTS cohort demonstrated that, in association with a diagnosis of PTLD, death from graft loss was as frequent as death from PTLD [81]. Therefore, the goal of PTLD treatment should be to induce disease regression while preserving graft function.

Pediatric T-cell PTLD cases seem to have a poor prognosis; Lundell et al. [14] reported 11 deaths among 14 patients. This type should be treated with the use of different chemotherapy regimens than those used to treat B-cell PTLD, such as intensive acute lymphoblastic leukemia-type treatment [15]. Most T-cell PTLDs are not EBV-related. Thus, reduction of immunosuppression may not be effective. It would be appropriate that combination chemotherapy be given since maintenance of immunosuppression is required for allograft protection. However, it will be necessary to collect information on a large number of cases to determine the optimal management strategy for T-cell PTLD.

Overall mortality of PTLD is difficult to assess, given the heterogeneity of the disease and underlying conditions;

however, estimates of approximately 40–70% have been reported after solid organ transplantation [12]. In contrast, after HSCT, disease progression is rapid with a greater incidence of fulminant, disseminated disease and an early mortality rate for PTLD approaching 90% [12, 83].

9 Prophylaxis

Antiviral agents, such as acyclovir and ganciclovir or valganciclovir, are often given at the first suspicion of PTLD and may have some role in the prophylaxis of PTLD [84, 85], although no study has yet demonstrated a clear benefit [86]. Ganciclovir is more effective against EBV-infected cells than acyclovir [87], but neither seems to be effective against late-onset or monomorphic PTLD [88]. No benefit is obtained by the addition of intravenous immunoglobulin to antiviral drugs [89, 90]. Another study using acyclovir combined with mycophenolate mofetil demonstrated a dramatic decrease in PTLD occurrence, implying a crucial role of mycophenolate mofetil in decreasing the incidence of PTLD [91].

The PSIs, everolimus and sirolimus, have both immunosuppressive and antiproliferative effects. Their activity is exerted through inhibition of the mammalian target of rapamycin (mTOR), resulting in inhibition of growth factor-induced proliferation of lymphocytes, as well as other hematopoietic and non-hematopoietic cells of mesenchymal origin. They have also been reported to inhibit the growth of EBV-transformed B lymphocyte in vitro and in vivo [38, 92]. mTOR-signaling pathways have been documented to be constitutively activated by lesional cells in PTLD [93]. PSIs might therefore be promising agents for preventing rejection and PTLD development [94]. CNI minimization with PSIs or conversion from CNIs to PSIs is currently being trialed, with the additional possibility of renal function preservation [95, 96]. The use of PSIs has been reported in association with pediatric PTLD [97, 98]. Further studies are needed for evaluation of the efficacy of PCIs.

A potential approach in EBV-seronegative pediatric recipients is EBV vaccination. However, no vaccine is currently available. One candidate vaccine uses recombinant gp 350, which is a viral antigen of the EBV capsid [19, 99]. The vaccine seems to prevent the development of EBV-related mononucleosis but not EBV infection [99]. Thus, its use is limited in post-transplant recipients.

10 Conclusion

PTLD remains a serious complication after pediatric heart transplantation, resulting from immunosuppression for prevention of allograft rejection. Most cases are EBV-related B-cell tumors. Primary EBV infection is highly associated with the development of PTLD in younger children. Treatment strategy is based on the reduction of immunosuppression, rituximab, or chemotherapy depending on the type of PTLD. Minimization of immunosuppression often causes death from allograft loss. Thus, the goal of PTLD treatment should be to induce disease regression while protecting the allograft.

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Antifungal Prophylaxis With Micafungin in Patients Treated for Childhood Cancer

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Background. Invasive fungal infections (IFIs) remain a major cause of infectious morality in neutropenic patients receiving chemotherapy or hematopoietic stem cell transplantation (HSCT). Micafungin exhibits broad antifungal activity against both *Aspergillus* and *Candida* species. We performed a retrospective study to determine the efficacy and safety of prophylactic micafungin against IFI in pediatric neutropenic patients during chemotherapy or HSCT. ***Procedure.*** Forty patients were given micafungin (3 mg/kg/day) intravenously for neutropenia: 131 patient-cycles (39 patients) after chemotherapy and 15 patient-cycles (14 patients) after HSCT. Median duration of neutropenia and micafungin prophylaxis was 13 and 23 days after chemotherapy and HSCT, respectively. ***Results.*** Treatment success rate, defined as absence of proven, probable, possible, or suspected IFIs, was 93.9% (121/131) and 80.0% (12/15) for chemotherapy and HSCT, respectively. Proven or probable IFI was documented in only one patient after HSCT. No adverse events were observed that could be related to micafungin prophylaxis. ***Conclusions.*** These results suggest that prophylactic micafungin is well tolerated and may prevent IFIs in pediatric patients with neutropenia receiving chemotherapy or HSCT. Pediatr Blood Cancer 2009;53: 605–609. © 2009 Wiley-Liss, Inc.

Key words: anitifungal prophylaxis; chemotherapy; children; hematopoietic stem cell transplantation; micafungin

INTRODUCTION

Invasive fungal infections (IFIs) remain a major cause of death in neutropenic patients receiving chemotherapy or hematopoietic stem cell transplantation (HSCT) despite the availability of antifungal agents. The risk of IFI is associated with the degree and duration of neutropenia, the disruption of mucosal barriers, and corticosteroid use. Fluconazole has been widely used as an antifungal prophylactic agent [1,2]. However, fluconazole does not protect patients from invasive asperigillosis and may cause emergence of resistant *Candida* species, that is, *C. krusei* or *C. glabrata* [3].

Micafungin is a new member of the echinocandins, which represent a new class of antifungal agents that non-competitively inhibits biosynthesis of 1,3-β-glucan linkages by interfering with 1,3-β-D-glucan synthase complex, an enzyme unique to fungi [4]. The 1,3-β-D-glucan target is an essential component for fungal cell wall synthesis [4]. Micafungin exhibits fungicidal activity against *Candida* species, including fluconazole-resistant isolates, and fungistatic activity against *Aspergillus* species [4,5]. A study evaluating micafungin versus fluconazole for prophylaxis during neutropenia after HSCT showed superior success rate with micafungin [6].

Micafungin has been available in Japan since 2002, and it has been used for treatment of asperigillosis and candidiasis as well as prophylaxis after HSCT. So far, there have been few reports describing its prophylactic use in pediatric patients [6]. The aim of this study was to evaluate the efficacy and safety of antifungal prophylaxis with micafungin in neutropenic children receiving chemotherapy or HSCT for childhood cancer.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the records of 40 children with neutropenia during chemotherapy or HSCT at Osaka University Hospital between May 2006 and September 2008. The details of medication for each patient were determined by review of the electronic chart system, which recorded all medications in our hospital. This study was approved by the Institutional Review Boards of Osaka University Hospital.

Antifungal Prophylaxis

If patients had an absolute neutrophil count <500 cells/μl during chemotherapy or HSCT, they received prophylaxis with micafungin 3 mg/kg once daily as a 1-hr infusion. All eligible neutropenic patients were given micafungin prophylaxis in the survey period. Five patients received antifungal prophylaxis after treatment failure with micafungin in previous cycles; one received micafungin again, while the remaining four discontinued micafungin prophylaxis and received another antifungal agent for prophylaxis. No patients suffered from IFI or received antifungal drugs before the start of micafungin prophylaxis. All patients received granulocyte colony-stimulating factor (G-CSF) or macrophage colony-stimulating factor (M-CSF). Micafungin prophylaxis was continued until recovery of absolute neutrophil count ≥500 cells/μl after the nadir absolute count or development of proven, probable, possible, or suspected IFI.

Outcomes

Treatment success was defined as the absence of proven, probable, possible, or suspected IFI during prophylaxis therapy. Proven, probable, or possible IFI was defined as described by EORTC [7]. Patients were considered to have proven infection if fungal elements were detected in biopsy specimens or cultures of supposedly sterile materials or blood. Patients were deemed to have probable IFI if fungal elements were detected directly or indirectly (galactomannan antigen or serum β-D-glucan) in conjunction with

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compatible clinical and radiographic findings. Possible IFI was defined if sufficient clinical evidence was consistent with IFI but without mycological support. Furthermore, we defined suspected IFI if patients had persistent fever ≥38°C for more than 48 hr despite broad-spectrum antibacterial therapy, when micafungin was replaced by or required addition of another antifungal agent.

Indirect mycological tests detecting antigen or cell wall constituents included serum galactomannan antigen (*Aspergillus* antigen) or serum β-D-glucan. Galactomannan antigen was measured using the Pastorex *Aspergillus* latex agglutination (LA) test (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) and its cut-off value was 15 ng/ml. Serum β-D-glucan was measured by a colorimetric assay (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and a cut-off value ≥7 pg/ml was used [8].

The following points were also determined: the duration of neutropenia, the time to IFI, mortality, and adverse events. Hematological and serum chemistry analyses were performed at least twice weekly, and fungal surveillance cultures at least once weekly.

RESULTS

A total of 40 patients (total 146 patient-cycles) received micafungin as prophylaxis for IFI. Patient characteristics are listed in Table I. Underlying diseases include acute leukemia (n = 16), non-Hodgkin lymphoma (n = 4), and solid tumors (n = 20). All but one patient with leukemia underwent chemotherapy and HSCT. Thirty-nine patients received micafungin in a total of 131 patient-cycles of chemotherapy. The number of cycles of chemotherapy was 1–9 per patient. Fourteen patients underwent a total of 15 HSCTs (one patient underwent HSCT twice): autologous HSCT (n = 5) and allogeneic HSCT (n = 10).

The median duration of neutropenia (<500 cells/μl) was 13 (range: 5–50) days for chemotherapy and 23 (range: 13–81) days for HSCT, while the median duration of micafungin prophylaxis for these groups was 12 (range: 5–49) days and 21 (range: 13–62) days, respectively. Successful prophylaxis was achieved in 123 of 131 patient-cycles (93.9%) for chemotherapy and 12 of 15 HSCTs (80.0%), and in 32 of 39 patients (82.1%) for chemotherapy and 11 of 14 HSCT patients (78.6%) (Table II). In total, 30 of 40 patients (75.0%) had successful prevention of IFI (Table II). One case, the only case where another course of micafungin prophylaxis was given after prior failure, showed repeated failure in the following course.

Cases of prophylaxis failure are summarized in Table III. Proven IFI was observed in only one patient, who received micafungin prophylaxis for 62 days for prolonged neutropenia. The patient had received micafungin prophylaxis for neutropenia after chemotherapy against disease relapse after autologous HSCT and, without recovery of neutropenia, subsequently underwent allogeneic HSCT with continued micafungin prophylaxis. Graft-versus-host disease (GVHD) prophylaxis was tacrolimus and short-term methotrexate; however, the patient developed grade III acute GVHD. In this case, *Candida parapsilosis* was detected in blood and stool cultures without abnormal radiological findings. No probable or possible IFI cases were observed.

Suspected IFIs were observed in 10 cases: eight after chemotherapy and two after HSCT. In all 10 suspected cases, neutropenia continued more than 14 days, and in eight of these cases, suspected IFI developed after more than 21 days of micafungin prophylaxis. Among these, one case had a positive result for galactomannan antigen, which was presumably associated with the repair of an air-conditioner. In another two cases, elevated serum β-D-glucan was documented within 1 week after switching antifungal agents: the highest level of serum β-D-glucan was 8.5 and 39.1 pg/ml in cases 7 and 9, respectively. All suspected cases showed no abnormal radiological findings.

For proven or suspected IFI cases, micafungin was replaced by or had the addition of another antifungal agent according to the guidelines of Slavin et al. [9]. Micafungin was switched to voriconazole in nine cases; in two of them, voriconazole was switched again to liposomal amphotericin B. In another two cases, liposomal amphotericin B was added to micafungin. All of these 11 cases were successfully treated with improvement of clinical and mycological findings.

We observed hepatic abnormalities during micafungin prophylaxis: AST, ALT, and total bilirubin elevations at grade 2 or higher according to National Cancer Institute-Common Toxicity Criteria

TABLE I. Patient Characteristics

	Chemotherapy		HSCT	
	Patient-cycle	Patient	Patient-cycle	Patient
Total no.	131	39	15	14
Median age, years (range)	6 (1–17)		5 (2–11)	
Underlying disease, n				
Leukemia	44	15	4	4[a]
Non-Hodgkin lymphoma	12	4	1	1[b]
Solid tumor	75	20	10[c]	9[b,c]
Duration of neutropenia, days				
0–7	6		0	
8–14	69		1	
15–21	28		6	
22–28	11		4	
>28	17		4	
Median (range)	13 (5–50)		23 (13–81)	

HSCT, hematopoietic stem cell transplantation. [a]Three patients underwent both chemotherapy and HSCT, while one patient underwent HSCT only; [b]All patients underwent both chemotherapy and HSCT; [c]One patient underwent HSCT twice.

TABLE II. Outcome of Prophylaxis

	Patient-cycle		Patient		
	Chemotherapy (N = 131)	HSCT (N = 15)	Chemotherapy (N = 39)	HSCT (N = 14)	Total (N = 40)
Development of IFI					
Proven	0	1	0	1	1
Probable	0	0	0	0	0
Possible	0	0	0	0	0
Suspected	8	2	7	2	9
Treatment success rate	123/131 (93.9%)	12/15 (80.0%)	32/39 (82.1%)	11/14 (78.6%)	30/40 (75.0%)

HSCT, hematopoietic stem cell transplantation; IFI, invasive fungal infection.

(NCI-CTC) occurred in 65.8%, 68.5%, and 6.2% of cases, respectively, (Table IV). These abnormalities were, however, considered to be related to chemotherapeutic agents and values normalized after the end of micafungin prophylaxis. No renal abnormalities were observed (Table IV). Thus, no adverse events were documented in association with micafungin administration.

DISCUSSION

The diagnosis of IFI is often delayed or difficult to establish with certainty, which can cause a delay in antifungal treatment and increased mortality [10,11]. Antifungal prophylaxis has therefore been commonly used as a treatment strategy. Micafungin has been demonstrated to exhibit excellent in vitro activity against both *Candida* and *Aspergillus* species [4], and clinical studies have also shown good activity in the treatment of IFI in patients with febrile neutropenia [12,13].

Our study evaluated micafungin as prophylactic antifungal therapy in an exclusively pediatric patient population. Treatment success was achieved in 123 (93.9%) of 131 of patient-cycles after chemotherapy and in 12 (80%) of 15 HSCT patients. Exclusion of the patients after treatment failure in previous cycles; however, might have affected the treatment success rate. A previous evaluation of micafungin versus fluconazole for prophylaxis during neutropenia after HSCT in a predominantly adult population showed superior overall efficacy for micafungin compared to fluconazole (80.0% vs. 73.5%, $P = 0.03$) [6]. Hashino et al. [14] reported a prophylactic success rate of 87.8% (none with proven or probable IFI) in 41 adult patients receiving micafungin after HSCT. The incidence of proven, probable, and possible IFI was reported at 13.6% in pediatric patients with leukemia who were receiving fluconazole during chemotherapy [15]. In a report describing the result of itraconazole prophylaxis in pediatric HSCT patients [16], itraconazole was discontinued prematurely in 11 (21%) of 53 patients because of IFI development (n = 2), fever (n = 7), fever plus veno-occlusive disease (n = 1), and toxicity (n = 1).

In our study, proven IFI was observed in one patient, in whom *C. parapsilosis* was detected in blood culture after 62 days of micafungin prophylaxis against prolonged neutropenia. *C. parapsilosis* is the second most common *Candida* species in the Asia-Pacific region and the third in Europe and North America [17]. Furthermore, *C. parapsilosis* is reported to be less susceptible to

TABLE III. Cases of Treatment Failure

IFI	Case no.	Therapy	Diagnosis	Age (years)	Duration of neutropenia (days)	Time to IFI (days)	Change of antifungal agents	Notes
Proven								
	1	Allo-HSCT	NBL	6	81	62	VRCZ → L-AmB	*C. parapsirosis* in blood culture
Suspected								
	2	Chemotherapy	NBL	6	15	9	VRCZ	Positive galactomannan antigen
	3	Chemotherapy	BT	16	17	14	VRCZ	
	4	Chemotherapy	ALL	9	22	22	VRCZ → L-AmB	
	5	Chemotherapy	AML	12	28	13	+ L-AmB	
	6	Allo-HSCT	NBL	5	28	21	+ L-AmB	
	7	Chemotherapy	ALL	4	30	20	VRCZ	[a]Elevated β-D-glucan (8.5 pg/ml)
	8	Allo-HSCT	NBL	3	31	21	VRCZ	
	9	Chemotherapy	ALL	4	31	19	VRCZ	[a]Elevated β-D-glucan (11.4 → 39.1 pg/ml)
	10	Chemotherapy	RMS	5	38	20	VRCZ	
	11	Chemotherapy	AML	12	38	23	VRCZ → L-AmB	

IFI, invasive fungal infection; NBL, neuroblastoma; ALL, acute lymphoblastic leukemia; BT, brain tumor; AML, acute myelocytic leukemia; RMS, rhabdomyosarcoma; VRCZ, voriconazole; L-AmB, liposomal amphotericin B. [a]Documented after changing of antifungal agents.

TABLE IV. Outcome of Adverse Effect

	Pre (N = 146)	Post (N = 146)	Most pathological value[a]
ASL (IU/L)	36.0 ± 25.2	30.4 ± 11.6	244 ± 123 (N = 96, 65.8%)
ALT (IU/L)	41.0 ± 65.8	34.8 ± 19.2	276 ± 123 (N = 100, 68.5%)
Total bilirubin (mg/dl)	0.53 ± 0.41	0.48 ± 0.26	2.49 ± 0.97 (N = 9, 6.2%)
Creatinine (mg/dl)	0.31 ± 0.10	0.30 ± 0.12	No abnormalities

AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are shown as mean ± standard deviation. [a]Most pathological values are shown for cases having grade ≥2 toxicity according to National Cancer Institute-Common Toxicity Criteria (NCI-CTC).

micafungin (MIC_{90} = 2 μg/ml) [17,18]. Breakthrough trichosporonosis has also been reported in patients receiving micafungin treatment [19]. Caution is therefore needed when employing prolonged prophylaxis with micafungin.

We started empirical antifungal therapy, that is, switching of antifungal agents, for persistent or relapsing antibiotic-resistant neutropenic fever during antifungal prophylaxis. Ten such cases in our study were defined as suspected IFI. Galactomannan antigen in serum has excellent specificity, but relatively low sensitivity for diagnosis of invasive asperigillosis [20,21]. One suspected IFI case had a positive result for galactomannan antigen by LA test in this study, although it might have been false positive. The patient thereafter received voriconazole without development of proven IFI. Serum β-D-glucan has also been reported to be a useful diagnostic tool for IFI [8,22–24]. However, a high proportion of false-positive results were documented, which still requires an optimal cut-off setting and repeated samplings [8,24]. In our study, we used an adapted cut-off value of 7 pg/ml, above which the best diagnostic performance has been reported to be obtained on two consecutive samples with positive predictive value of 0.79 and negative predictive value of 0.91 [8]. At diagnosis of suspected IFI, no cases were found to have elevated β-D-glucan in our study. In two cases; however, β-D-glucan was increased after changing antifungal agents, but later decreased without any clinical or radiological signs of IFI, which indicates that continued administration of alternative drug (voriconazole in these cases) led to improvement.

The safety of micafungin has been reported in patients with febrile neutropenia [6,25] and in pediatric patients [26]. Micafungin was similarly well tolerated in our study, as demonstrated by the lack of documented adverse effects. It has been reported that the administration of micafungin at a daily dose of 100 mg is promising for prophylactic antifungal therapy in adult patients undergoing allogeneic HSCT [14]. However, age-related differences in pharmacokinetic parameters have been reported in pediatric patients with febrile neutropenia: mean total body clearance was higher in patients <8 years than in those ≥8 years (0.385 vs. 0.285 ml/min/kg) [26]. That study also showed no dose-limiting toxicity for micafungin up to 4 mg/kg/day in febrile neutropenic pediatric patients [26]. Therefore, the dose of 3 mg/kg, used in our study, appears to be acceptable for efficacy and safety.

The use of micafungin was not associated with nephrotoxicity or infusion-related reactions, which are commonly observed in patients receiving amphotericin B [27]. The pharmacokinetics of micafungin were not altered by concomitant administration of tacrolimus, mycophenol mofetil, prednisolone, or amphotericin B, since micafungin appears to be metabolized primarily through the *O*-methyl transferase pathway and minimally through the cytochrome P450 3A pathway [5]. The pharmacokinetic profile of micafungin makes it highly suitable for the HSCT setting. The absence of drug–drug interactions is a major consideration for the use of micafungin in patients who are considered as needing systemic antifungal prophylaxis in vincristine-containing treatment protocols [28]. Moreover, animal studies showed significant synergy for micafungin with liposomal amphotericin B or itraconazole [29–31]. All these factors, combined with its efficacy, make micafungin an attractive choice for prophylaxis of IFIs in neutropenic pediatric patients.

In summary, micafungin is an effective and well tolerated option for antifungal prophylaxis in neutropenic pediatric patients undergoing chemotherapy or HSCT. A randomized study is needed to confirm the efficacy of this approach.

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