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TRANSPLANTATION

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Epstein-Barr virus monitoring in paediatric renal transplant recipients

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Abstract Prospective Epstein-Barr virus (EBV) surveillance post transplant was undertaken by qualitative polymerase chain reaction testing for EBV DNA in plasma so as to detect EBV viremia as early as possible and thereby attempt to pre-empt post-transplant lymphoproliferative disease by reduction of immunosuppression. Forty-three children (46 transplants) were followed for a median (range) of 15.5 (3–25) months. Thirty-one children (67%) were EBV seropositive pre transplant. Twenty children (44%) developed EBV viremia; of these 9 (60%) were seronegative and 11 (36%) seropositive recipients. Primary infection developed later **(**median difference 14.2 weeks, *P*=0.009), was more likely to be symptomatic (odds ratio 2.91, 95% confidence interval 0.95–4.88) and associated with a rise in serum creatinine (odds ratio 6.13, 95% confidence interval 4.13–8.13) than reactivation disease. There was a higher incidence of EBV disease in children receiving quadruple therapy and tacrolimus (odds ratio 13.2, 95% confidence interval 11.5–14.9) compared with those given cyclosporin-based immunosuppression. Immunosuppression was reduced when EBV infection was detected. All children became asymptomatic and renal function returned to normal by a median (range) of 17 (6–52) days, although mild relapses occurred in 3 children. Regular EBV surveillance allowed prompt reduction of immunosuppression and was associated with a good outcome in this group of children.

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Introduction

Epstein-Barr virus (EBV) is an oncogenic herpes virus that is associated with a number of lymphoid and epithelial cell malignancies and lymphoproliferative diseases in the immunocompromised host [1, 2]. The virus infects most people in early childhood and thereafter remains in the body of the individual, usually as a latent infection [3].

Of episodes of unexplained fever and/or steroid-resistant rejection in children with a renal transplant, 60% are caused or complicated by viral infections – cytomegalovirus (CMV), EBV, varicella, parvovirus B19 and adenovirus [4]. With the use of more potent immunosuppression, such infections are seen with increasing frequency. The term post-transplant lymphoproliferative disorder (PTLD) describes a spectrum of lesions that occur in transplant recipients in association with primary EBV infection or EBV reactivation. The incidence is highest in the 1st post-transplant year (up to 20-fold higher) [5], and is particularly common in EBV-seronegative children (up to 200-fold higher) [6]. PTLD in its mild forms may resolve with reduction of immunosuppression [7]. However, this may lead to impairment of graft function or to graft loss [8, 9]. In its more severe forms, as with lymphomas, chemotherapy may be necessary, and mortality is high [10].

We commenced routine post-transplant screening for EBV DNA by polymerase chain reaction (PCR) in January 2000. This enabled us to detect EBV infection as early as possible in the post-transplant course, before the development of symptoms and at a time when modification of immunosuppression might preempt the development of overwhelming infectious mononucleosis or frank malignancy. We report the incidence, timing, symptomatology, effect on graft function and outcome of EBV disease post transplant.

Patients and methods

Patients

Forty-six renal transplants were performed in 43 children (24 boys and 19 girls) between January 2000 and December 2001. Thirty-seven patients (81%) received their first transplant, 8 (17%) their second transplant and 1 (2%) their third transplant. Of these, 3 children received two transplants during the study period. The mean (range) age at transplantation was 11.3 (2.2–18.3) years. Of the 46 transplants 13 (28%) were from living-related donors. Twenty-three (53%) children had renal dysplasia with or without obstruction or reflux; 11 (26%) congenital nephrotic syndrome; 3 (7%) had nephrectomies for Wilms tumour; 2 each had cystinosis, nephronophthisis and autosomal recessive polycystic disease; and 1 each had atypical hemolytic uremic syndrome (HUS), cortical necrosis and unknown cause. All but 2 patients, who left the country after 3 months, were kept under regular follow-up for a median (range) duration of 15.5 (3–25) months with clinical and laboratory monitoring for EBV infection.

All children receiving first transplants were treated with steroids, azathioprine and cyclosporin (n=32, 70%) until February 2001. After this, as part of a randomised trial, patients received either cyclosporin (n=2, 4%) or tacrolimus (n=2, 4%) and Basiliximab in addition to steroids and azathioprine. Recipients of second or subsequent transplants (n=9) were managed with quadruple immunosuppression comprising steroids, azathioprine, cyclosporin A and anti-thymocyte globulin (n=5) or Basiliximab (n=2); or steroids, azathioprine, tacrolimus and Basiliximab (n=2). Anti-thymocyte globulin was given to 5 patients (11%) and Basiliximab to 4 (9%) of second or subsequent graft recipients. These immunosuppression regimens have been subsequently referred to as triple or quadruple immunosuppression. One patient with HUS was treated with Basiliximab, steroids and mycophenolate mofetil. Cotrimoxazole was routinely given to all patients for *Pneumocystis carinii* prophylaxis. We do not give CMV prophylaxis, but screen weekly for CMV PCR positivity and treat with ganciclovir if this develops.

Rejection was diagnosed by a rise in creatinine of greater than 10% on two successive readings in the absence of other potential causes. Unfortunately, rejection was not always biopsy proven due to difficulties with the service at that time. Rejection episodes were treated with methylprednisolone intravenously at 600 mg/m^2 for 3 days in the first 6 weeks post transplant and prednisolone orally at 3 mg/kg for 3 days thereafter.

During the study period 5 children (10.9%) lost their graft (3 had vascular complications, 1 had a recurrence of focal segmental glomerulosclerosis in the graft and 1 rejection). There was no graft loss or death through EBV-related disease or treatment.

Of the 46 donors, 39 (85%) were CMV IgG positive, while 34 (74%) of the recipients were CMV IgG positive pre transplant. As the EBV status of the donor is not routinely checked in the United Kingdom, in our study the EBV serostatus of only 4 live donors was known; 3 of them were EBV positive as confirmed by IgG tests. One immunologically naïve patient received an EBV-positive kidney; clinically symptomatic disease with early graft rejection was seen.

Methods

All children had their EBV and CMV serostatus determined pre transplant. Sera were tested for IgG and IgM antibodies against EBV capsid antigen (VCA) by immunofluorescence (Bion, supplied by Ati Atlas, Chichester, UK). A gelatin particle agglutination test (Serodia-CMV, Mast Diagnostics, Bootle, UK) was used to detect the presence of CMV antibodies (IgM and/or IgG) and an enzyme immunoassay for CMV-specific IgG (Bioelisa CMV IgG, Biokit UK**)**. If negative, tests were repeated at 3-monthly intervals in children awaiting a cadaveric donor transplant.

The day after transplantation, blood was sent for EBV and CMV PCR; then weekly for the first 3 months, and then monthly for the 1st year. EBV and CMV PCR were also requested at the time of pyrexial illnesses and episodes of graft dysfunction.

Qualitative EBV and CMV PCR

DNA was extracted from 200 µl of whole blood in EDTA or plasma using the QIAamp DNA blood minikit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. For EBV detection, a PCR using primers IR1a-5′ and IR1a-3′ that amplify a specific region of 288 base pairs within the internal repeat (IR) was performed [11]. Negative and positive controls were included in each run. If EBV DNA was detected in whole blood, the test was repeated the following day on the extracts of whole blood and plasma. The quantification step was not performed, but the threshold of detection of the assay was found to be approximately 100 DNA copies/ml when samples were run in parallel with an external laboratory that uses a light cycler for quantification and EBV-infected cells (10 genomic EBV DNA copies/cell). The presence of EBV DNA was only regarded as clinically significant if it was detected in whole blood and plasma, i.e., >100 copies/ml plasma.

PCR for the detection of CMV DNA was performed on the same extracts of whole blood as used for EBV; 5 µl of extracted DNA was added to a standard PCR reaction mixture containing 1× PCR Buffer II, 2 mM $MgCl_2$, 100 ng each of sense gB1 (81638–81707) and antisense gB2 (81580–81558) primers [12] and 1 unit of Amplitaq Gold (Perkin Elmer, Warrington, UK). The PCR has a threshold of detection of approximately 2,000 CMV DNA copies/ml.

PCR was performed in a Hybaid, Omnilab thermocycler. An initial denaturation at 95°C for 10 min was followed by 94°C (30 s) denaturing, 62°C (30 s) annealing and 72°C (30 s) extension for 40 cycles, with a final extension at 72°C for 5 min. PCR products were run on 2% agarose gels, containing ethidium bromide, for 30 min at 120 mA and visualised on an ultraviolet transilluminator.

An active EBV infection was defined as EBV DNA positive in plasma but not in blood, thereby indicating a viral load >100 copies/ml. Detection of EBV DNA in plasma of a previously seronegative patient (VCA IgM and/or VCA IgG negative) was considered to be indicative of primary infection. A reactivation of EBV was recorded when EBV DNA was detected in blood and plasma of recipients who were EBV seropositive prior to transplantation. We did not test for IgM positivity post transplant, as all the patients were given blood, which may affect the results.

We defined tonsillar enlargement, cervical lymphadenopathy, elevated hepatic enzymes and splenomegaly as infectious mononucleosis. If there was evidence of lymphadenopathy elsewhere or involvement of other systems then we would investigate for PTLD, including biopsy, screening for EBV in the biopsy and full radiological assessment. This was not indicated in any of the children in this study.

Data collection

The following data were collected: time to EBV seroconversion, clinical events surrounding an episode of EBV seroconversion, changes in serum creatinine associated with seroconversion, changes in immunosuppression associated with seroconversion and episodes of graft rejection.

Statistics

Groups were compared using the chi-squared test and the odds ratio with 95% confidence intervals. Non-parametric data (the comparative effect of different immunosuppressants) were analysed using the Mann-Whitney U test.

Results

EBV status pre transplant

Of the 46 transplant recipients, 31 (67%) were EBV IgG positive pre transplant. The median (range) age of the EBV positive group was 14.8 (2.4–18.3) years, which was significantly higher than the seronegative group [10.3 (3.25–16.6) years], (P=0.03). The EBV status according to age is shown in Table 1.

Incidence and timing of EBV infection – primary and reactivation disease

Of the 46 transplant recipients, 20 (44%) had EBV viremia post transplant. Of the 20 children, 9 (45%) were EBV seronegative pre transplant, indicating a primary EBV infection, while 11 children (55%) had reactivation of the virus post transplant (Table 2). Thus, 60% of the pre-transplant EBV-negative children developed primary EBV infection, whereas only 36% of the pre-transplant EBV-positive group developed a reactivation of the disease post transplant.

Primary EBV infection developed later (P=0.009) than reactivation of virus. The median time to disease post transplant was 20.6 (range 6–61) weeks in the group with primary EBV disease compared with 7.4 (range 1.6–2.3) weeks in those with EBV reactivation.

Table 1 The pre-transplant Epstein-Barr virus (EBV) status of the study population according to age

Age range (years)	*n*	No. seropositive (%)
<5	8	2 (25)
5–10	20	14 (70)
11–15	12	10 (83)
>15	6	5 (83)
Total	46	31 (67)

Clinical features

Of the 20 children, 13 (65%) were clinically symptomatic (Table 3). Children with primary infection were more likely to present with symptoms (odds ratio 2.91, 95% confidence interval 0.95–4.88) than those with virus reactivation. Eleven children had infectious mononucleosis with fever and ‘flu-like symptoms, 4 children had diarrhoea and 3 developed a blanching maculopapular rash. Five (38.4%) of the symptomatic patients had tonsillar enlargement and cervical lymphadenopathy. Oral ulcers, pneumonia and haematuria were seen in 1 case each. In 10 of the 13 symptomatic patients, EBV DNA was detected in plasma prior to the onset of clinical signs or symptoms; the median interval between PCR positivity and presentation was 5 (range 1–11) days.

Routine screening revealed EBV viremia in 7 asymptomatic children. Primary infection occurred in 2 cases; both had elevated liver transaminase enzymes at the time of diagnosis that reverted to normal within 2–3 weeks of reducing immunosuppression. In contrast, there were no biochemical features of infection in the 5 asymptomatic children with reactivated EBV.

Immunosuppression

The effect of baseline immunosuppression is shown in Table 4. There was a significantly higher risk of primary and reactivation EBV (odds ratio 13.2, 95% confidence interval 11.5–14.9) in the groups receiving tacrolimus or quadruple therapy compared with the group receiving cyclosporin-based immunosuppression.

When primary EBV infection was detected, immunosuppression was reduced in all but 2 cases. In these 2 patients, who had mild infectious mononucleosis-like symptoms and normal graft function, a “wait and watch” policy was taken, with close monitoring of clinical signs and serum creatinine levels. In the other 7 patients, azathioprine was halved and then stopped 2 weeks later if there was no improvement in clinical signs or graft function. Patients with reactivation of EBV did not have any reduction in immunosuppression unless there was evi-

Table 2 Incidence and timing of EBV infection – primary disease and disease reactivation

EBV infection	*n*	Total	%	Age, median (range), years	Conversion to EBV positivity, median (range), weeks (P=0.009)
Primary	9	15	60	9.5 (3.3–16.6)	20.6 (6–61)
Reactivation	11	31	36	12.0 (2.4–18.3)	7.4 (1.6–2.3)

Table 3 Clinical features, graft function and changes in immunosuppression

EBV infection (*n*)	Symptomatic	Rise in creatinine[a]	Reduction in immunosuppression
Primary (9)	7 (78%)	7 (78%)	7 (78%)
Reactivation(11)	6 (56%)	4 (36%)	3 (27%)

[a] An increase in creatinine of >10% of baseline level

Table 4 Immunosuppression used and the correlation with clinical events (*CyA* cyclosporin A, *Aza* azathioprine, *Pred* prednisolone, *ATG* anti-thymocyte globulin, *MMF* mycophenolate mofetil)

Immunosuppression	Total no.	No. seronegative pre transplant	No. becoming EBV positive (%)	No. seropositive pre transplant	No. of reactivations post transplant (%)
Triple – CyA+Aza+Pred	32	10	6 (60)	22	4 (18.2)
Triple – Tacrolimus+Aza+Pred	2	1	1 (100)	1	1 (100)
Quadruple – ATG+CyA+Aza+Pred	5	1	1 (100)	4	4 (100)
Quadruple – Basiliximab+CyA+Aza+Pred	4	2	1 (50)	2	2 (100)
Quadruple – Basiliximab+Tacrolimus+Aza+Pred	2	0	0 (0)	2	1 (50)
Basiliximab+MMF+Pred	1	1	0 (0)	0	0
Total	46	15	9	31	11

dence of graft dysfunction (rising creatinine or biopsy proven rejection) or associated CMV infection. Consequently, a greater proportion of patients with primary EBV infection (odds ratio 9.33, 95% confidence interval 7.17–11.55) was managed with a reduction in immunosuppression. Immunosuppression once reduced was not restarted.

Children with primary infection were at significantly greater odds (odds ratio 6.13, 95% confidence interval 4.13–8.13) of having a rise in serum creatinine from baseline levels of >10% [median (range) baseline creatinine level 54 (38–76); median (range) rise in creatinine 154 (78–285) at the time of EBV infection]. Resolution of symptoms occurred in all patients by a median (range) of 17 (6–52) days and creatinine returned to baseline in all patients by 11 (4–39) days. Following a reduction of immunosuppression the median (range) time to disappearance of EBV DNA in plasma was 2.1 (1.4 to 4.2) weeks.

Effect of treatment for rejection

Thirty-two children (70%) were treated for rejection; 14 of these (43%) had EBV infection post transplant; none of these cases developed following treatment for rejection. There was no significant difference (P=0.62) in the incidence of EBV infection between children treated for rejection and those who were not.

Simultaneous CMV infection

Three patients, who were CMV negative pre transplant, developed simultaneous CMV and EBV infection (confirmed by qualitative PCR for CMV DNA). All 3 patients had CMV-positive donors. These children had an earlier onset of infection [median (range) 5 (4.2–6) weeks post transplant]; the median (range) time to EBV DNA positivity in the group of patients without CMV infection was 14.2 (1.2–64) weeks. These patients were treated with a reduction in immunosuppression (azathioprine reduced and then stopped in 2 patients) and intravenous ganciclovir for 3 weeks. In all 3 patients the creatinine returned to baseline, and they made an uneventful recovery.

Follow-up

Three patients had clinical reactivation with infectious mononucleosis and EBV DNA positivity in plasma during the study period. The second infection occurred following a median time interval of 36 weeks (range 12–76 weeks) following the first infection and 44 weeks (range 28–81 weeks) post transplant. These later infections were generally less severe – 2 of the patients had enlarged tonsils and lymphadenopathy, while 1 patient had chronic fatigue and weight loss. Immunosuppression, once modulated, was not restarted in any of the patients. There was no rise in creatinine or evidence of graft rejection in any of these patients.

Discussion

This is the first published study to show that EBV surveillance post transplant, and prompt reduction of immunosuppression when EBV DNA is detected in plasma, are associated with a good outcome for the patient and the graft. This is important because failure to control EBV viral replication can lead to PTLD, which has a mortality of 50% and leads to graft loss in 63% of cases [13]. The incidence of PTLD in allograft recipients has been reported as 1%–4%, 2%, 2%–10% and 19% for adult kidney, liver, heart-lung and intestine transplants respectively [14, 15], although most figures are based on relatively small series from individual institutions.

In our series approximately two-thirds of the children were EBV seropositive pre transplant. This figure is higher than reported in other paediatric series, where it has been reported as 49% [16] and 30% [17]. Many of our patients were from inner city areas and ethnic minorities, where the incidence of EBV seropositivity is known to be higher. The high incidence of EBV seropositivity may have contributed to the good outcome in our patients. Younger children are more likely to experience primary EBV infection post transplant, and this has been associated with a higher morbidity [18]. The number of patients in this study was small, resulting in inadequate power to detect a difference in disease severity between younger and older patients (seropositive and seronegative groups).

The clinical manifestations of post-transplant EBV-related infections are heterogeneous and range from an infectious mononucleosis-like illness to monoclonal B-cell proliferation with nodal and extra-nodal tumours [19]. There are no uniform criteria, including both clinical and histological parameters, for the diagnosis of PTLD [15]. The myriad of clinical presentations, as well as a significant number of asymptomatic infections, calls for routine EBV testing in this highly susceptible population. The children in our study only had symptoms of infectious mononucleosis; i.e., fever, tonsillar enlargement and cervical lymphadenopathy. None had evidence of possible PTLD, such as chest involvement, lymphadenopathy elsewhere or systemic involvement, hence biopsies were not performed. However, we would recommend biopsy in children with such symptoms, and therefore suspected PTLD, or children with infectious mononucleosis-like symptoms that fail to improve promptly with reduction of immunosuppression.

The level of immunosuppression is known to be an independent risk factor for EBV-related complications [20]. From our series, active EBV infection was 13 times more likely with the use of tacrolimus or quadruple immunosuppression compared with cyclosporin; however as there were few transplants in different groups of intensified immunosuppression, larger studies would be required to demonstrate a statistical significance. Tacrolimus-based immunosuppression is particularly implicated in this process [21, 22]. In contrast, the use of mycophenolate mofetil in a steroid-free immunosuppressive protocol was associated with a lower incidence of primary infection or reactivation of EBV [23]. As most patients in our study were immunosuppressed with cyclosporin, this may explain our lower incidence of EBV-related complications compared with other groups. There was a high incidence of treatment for rejection in our patients, but we found no association between treatment for rejection and EBV disease. However, the failure to show such a relationship may have been due to small patient numbers.

Once the diagnosis of an EBV-related infection is made, treatment strategies remain ill-defined. A reduction in immunosuppression so as to allow sufficient recovery of the host immune response to control lymphoproliferation, hopefully without causing rejection of the allograft, is recommended in most cases [20]. In our study, reduction of immunosuppression was associated with clinical recovery and normalization of graft function in all cases, without later graft loss due to rejection. The early detection of infection by routine EBV screening followed by prompt reduction in immunosuppression may explain the good clinical outcome in our patients.

CMV disease has been shown to be associated with an increased risk of PTLD in both adult and paediatric transplant recipients [16, 24]. For CMV-seronegative recipients receiving a CMV-positive allograft, the risk of PTLD increases four- to sixfold [24]. In our study, simultaneous CMV and EBV infection resulted in an earlier onset of infection and more severe symptoms, although none of the patients progressed to develop the clinical spectrum of PTLD.

Antiviral agents were not used in our study. Some studies suggest that these may have a role in modulating the severity of EBV infection [25, 26], but neither intensive nor prolonged treatment with antiviral agents like ganciclovir or aciclovir affects the incidence of EBV infection in post-transplant seronegative patients [27, 28]. We have no experience with the use of newer antiviral agents such as valganciclovir in our patients, and have been unable to find any published data on its benefits in EBV DNA-positive renal transplant recipients.

PTLD is remarkable for a short post-transplantation time to onset – approximately 47% of cases occur within 6 months, 62% within 1 year and 90% within 5 years of transplant [29]. On follow-up none of our patients went on to develop complications – all maintained good graft function and there were no cases of EBV-related graft loss or patient morbidity in this group. However, 3 patients developed a reactivation of infection with clinical symptoms. The serum creatinine remained stable and good graft function was maintained. The significance of persistent low-grade viraemia on graft function and survival is not known. Larger studies with long-term follow up are required.

The primary goal of this study was to preempt PTLD before viral loads became high. The results show that no patient had any problems with graft rejection as a consequence of reduction of immunosuppression. A recent study has shown that no particular level of viral load consistently correlated with PTLD [30]. However, the introduction of quantitative PCR for EBV DNA will enable us to measure the effect of a reduction in immunosuppression in future studies. None of our patients were clinically felt to have PTLD, therefore it was not considered ethical to perform histopathological studies to establish whether or not this was the case.

In conclusion, routine qualitative screening for EBV infection in the immunocompromised host followed by prompt management with a reduction of immunosuppression plays an important role in the prevention of severe EBV-related problems. As reactivational disease is less severe than primary disease, the use of an EBV vaccine in seronegative patients might further reduce the incidence and severity of the disease. Long-term studies are required to understand the significance of prolonged low-grade EBV viraemia and reactivation of infection on long-term graft survival and late PTLD.

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