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Epstein-Barr Virus Transmission via the Donor Organs in Solid Organ Transplantation: Polymerase Chain Reaction and Restriction Fragment Length Polymorphism Analysis of IR2, IR3, and IR4

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Two organ transplant recipients who received organs from a common donor and were diagnosed as having an Epstein-Barr virus (EBV)-associated posttransplant lymphoproliferative disorder were studied to determine the mode of EBV transmission. The results of restriction fragment length polymorphism, polymerase chain reaction, and minisatellite DNA analyses demonstrate that both patients had a common strain of EBV and that this strain was transmitted from the donor's organs to both recipients. Posttransplant lymphoproliferative disorder resulted from the proliferation of EBV-immortalized B lymphocytes of the recipient, not those of the donor.

Epstein-Barr virus (EBV), a B-lymphotropic human herpesvirus, is the etiological agent of infectious mononucleosis and is strongly associated with Burkitt's lymphoma and nasopharyngeal carcinoma (3, 12, 13). EBV has been implicated in the pathogenesis of posttransplant lymphoproliferative disorders (PTLD), particularly in patients who develop primary EBV infections when immunosuppressed (10). PTLD may be defined as a series of disorders which vary from a severe polyclonal mononucleosis-like syndrome, which is widely infiltrative and may have a fatal outcome, to frank polyclonal or monoclonal B-cell lymphoma (8, 10, 15). Since patients contracting primary EBV infections within the first 3 or 4 months after transplantation are at greatest risk for the development of PTLD (9, 18), virus-host interactions during immunosuppression clearly play a critical role.

One previously unanswered question in the pathogenesis of PTLD concerns the reservoir of infectious virus and the mode of transmission during transplantation. There have been recent reports which suggest that the oropharyngeal cavity may not be the only reservoir of infectious virus but that lymphocytes may, in fact, be responsible for viral persistence. Yao et al. (20) observed that prophylactic acyclovir has little effect on the numbers of transformed B cells present in the peripheral blood. Gratama et al. also found that EBV infection in bone marrow recipients may be eradicated or replaced by the virus strain of the donor (6, 7). Similarly, the lesions of bone marrow recipients who subsequently develop PTLD frequently contain both donor and recipient cells (21). These observations suggest that a source of infection might be the donor, and they raise a second question of whether PTLD in solid organ recipients is a function of proliferating donor B cells and, if not, of whether the lymphocytes and possibly other cell types present in the donor organ can transmit the virus. The availability of two PTLD patients who had a common organ donor provided us with a unique opportunity to address the questions raised above. The two patients, termed T and P, received organs from a common donor. Patient T, who was EBV seronegative prior to heart-lung transplantation, developed a primary EBV infection about 1.5 months later, and a lung biopsy obtained 54 days after transplantation showed PTLD. Patient T died 80 days after transplantation. Patient P was seropositive for EBV prior to kidney transplantation. PTLD in the kidney was diagnosed following nephrectomy 64 days after transplantation. Peripheral blood was obtained from both patients concomitant with the diagnosis of PTLD.

The linear EBV genome consists of four internal repeats (IR1 to IR4) and two terminal repeat regions which are separated by five stretches of unique sequence (U1 to U5) (Fig. 1). According to the studies of Lung et al., the most polymorphic regions of the EBV genome lie within IR2, IR3, and IR4, which are located within the *Bam*HI H, K, and B′/I regions of the viral genome, respectively (14). When EBV DNA is assayed by restriction fragment length polymorphism (RFLP) analysis, the polymorphism of these regions can be attributed primarily to varying numbers of repeats contained within each region. Therefore, RFLP analysis of the *Bam*HI H, K, and B′/I regions and of IR2, IR3, and IR4 can provide an accurate means by which to distinguish EBV strain differences.

To analyze the EBV strain(s) present in both patients' peripheral blood lymphocytes (PBL), we needed to first isolate representative populations of EBV-transformed lymphocytes. Lymphoblastoid cell lines (LCL) were established from PBL by limiting dilution. Mononuclear cells were isolated from peripheral blood by centrifugation through Ficoll-Hypaque and seeded at decreasing concentrations of 2×10^5, 1×10^5, and 0.5×10^5 cells per 0.2 ml (final volume) in flat-bottomed 96-well dishes and at a concentration of 3×10^6 cells per culture in RPMI 1640 containing 10% fetal calf serum, 125 U of penicillin per ml (Sigma), 125 μg of streptomycin per ml (GIBCO), and 0.5 μg of cyclosporine per ml (Sandoz). Fifteen LCL were derived from the PBL of patient T, eight of which were clonal ($<2 \times 10^5$ peripheral mononuclear cells per culture). One LCL was developed from patient T's PTLD lesion by the direct outgrowth of a section of lung PTLD lesion tissue in RPMI 1640 containing 10% fetal calf serum, 5 μg of cyclosporine per ml, and antibiotics. Several clonal LCL were derived from the PBL of patient P and were pooled for EBV strain analysis. Total

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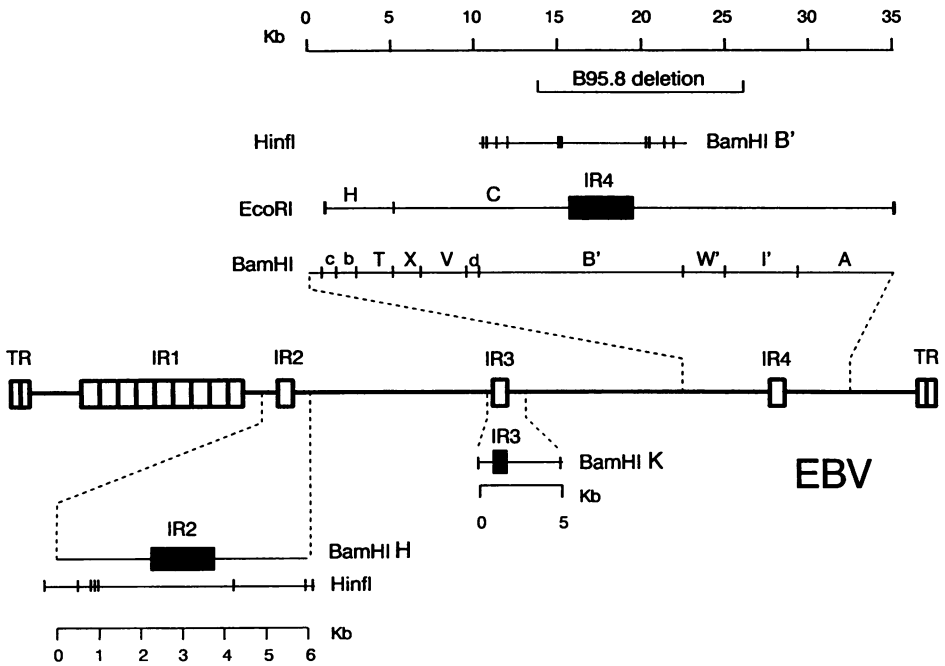

FIG. 1. Schematic of the EBV genome depicting the regions relevant to this study. Shown are the location and identity of the relevant *Bam*HI and *Eco*RI restriction fragments making up the polymorphic repeat regions of the viral genome (IR2, IR3, and IR4) (14). Included are expanded restriction maps of the *Bam*HI H and B' regions showing the location of the *Hin*fI sites. TR, Terminal repeat.

cellular DNA was extracted from the LCL and the PTLD lesion tissue and was analyzed by Southern blotting according to the modification described by Chen et al. (2). The results of this analysis can be summarized as follows: 16 LCL from patient T contained EBV DNA which was indistinguishable when digested with *Bam*HI and probed with plasmid DNA containing EBV sequences which span IR2, IR3, and IR4, i.e., the *Bam*HI H and K and the *Eco*RI C fragments of the viral genome, respectively (gifts of E. Kieff) (data not shown). To confirm that polymorphisms were not present within the most polymorphic region of the viral genome, IR2, DNA from all 16 LCL was digested with *Hin*fI, which has a number of sites within the *Bam*HI H region (Fig. 1), and was hybridized to the *Bam*HI H probe (data not shown). Again, we found all 16 cell lines to be indistinguishable at this level of analysis. Therefore, we concluded that patient T had a single strain of EBV present in both his PBL and his PTLD tumor lesion. A representative analysis of *Bam*HI-digested LCL DNA probed with the *Bam*HI H and K regions of the viral genome, comparing the pooled LCL with a single clonal line, is shown in Fig. 2. Analysis of patient P's early passage LCL demonstrated that at least two populations of EBV were present. This conclusion was based on the presence of two equal-molarity bands representing the *Bam*HI K and H regions of the EBV genome (Fig. 2). The polymorphism within the *Bam*HI H region was also analyzed by Southern blotting of *Hin*fI-restricted LCL DNA. Polymorphisms were present in both the 3.2-kb IR2 containing *Hin*fI fragment and in a fragment comprising the left end of the viral *Bam*HI H region (Fig. 1; data not shown). Interestingly, the analysis of the *Bam*HI- and the *Hin*fI-restricted DNA demonstrated that one of the two strains of EBV present in patient P's pooled LCL was indistinguishable from the single strain previously characterized in patient T (data not shown). The only detectable polymorphism between the two genomes of the putatively shared strain of EBV was found in the *Bam*HI B' region when the RFLP analysis was extended to include the complete *Eco*RI C/H region of the viral genome. It is doubtful whether this polymorphism represents a true strain difference, since it was not present within IR4 itself or in the regions which flank the internal repeat (data not shown). Considering the lack of polymorphism in IR2, IR3, and IR4 between the two viruses, this difference is more likely a mutation resulting from passage in vitro, similar to the deletions observed in the B95.8-derived LCL IB4 and X50.7 (19). Since each strain of EBV has a characteristic number of repeats within the IR2, IR3, and IR4 regions and since the two viral DNAs were indistinguishable in these regions, it is very likely that both patients share a strain of EBV. Western blot (immunoblot) analysis with an EBNA2-specific monoclonal antibody, PE2 (provided by E. Kieff), further supported the view that the two viruses are identical, since patient P's cells contained two easily distinguishable EBNA2 bands, one of which comigrated with the single EBNA2 band present in patient T's cells (data not shown). It is likely that the second strain of virus present in the PBL of patient P was acquired prior to transplantation, since this individual, in contrast to patient T, was EBV seropositive prior to transplant.

As mentioned above, the classical EBV infection route is through oral contact. However, the strain of virus present in patient P's throat wash LCL, derived by the transformation of cord blood lymphocytes with the filtered throat wash, differed both from strains present in patient P's PBL-derived LCL and from the strain present in patient T. The EBV strain present in patient P's throat wash contained a *Bam*HI H fragment of unique size (Fig. 2) and a polymorphism in *Bam*HI A and B' (data not shown). The polymorphism within the *Bam*HI H region mapped to the 3.2-kb IR2 region

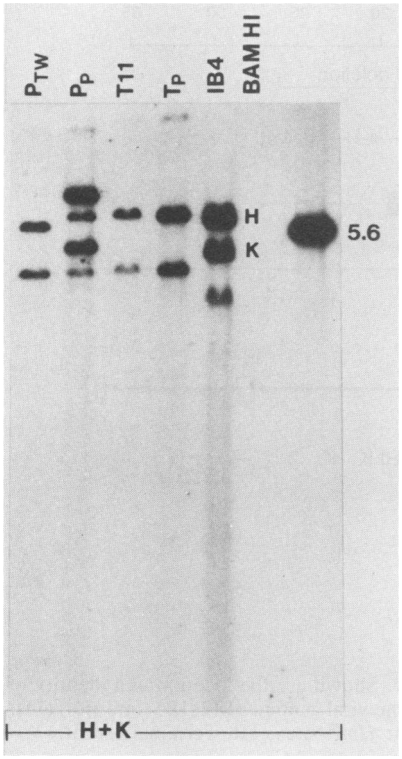

FIG. 2. Southern blot analysis of cellular DNA obtained from LCL derived from patients T and P, showing that patient P harbors multiple strains of EBV. The cellular DNA was digested with *Bam*HI and probed with plasmids containing both the *Bam*HI H and K regions of EBV. The locations of the relevant fragments in IB4 are indicated (note that the additional band present in this lane is pBR322 marker DNA). T_P and P_P, Pooled LCL derived from the peripheral blood of patients T and P, respectively; T11, LCL derived from patient T; P_{TW}, LCL derived from the infection of cord blood lymphocytes with patient P's throat wash. Concentrations of DNA loaded ranged from 3 to 15 μg. Number at right indicates kilobases.

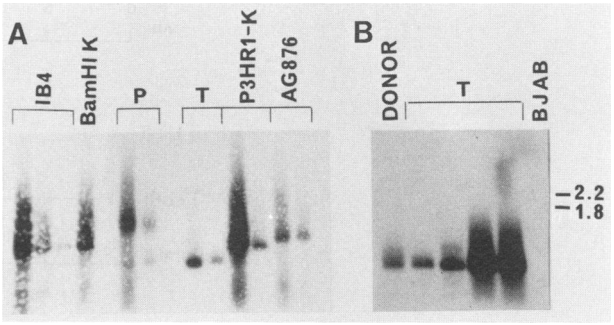

FIG. 3. Southern blot analysis of IR3 PCR-amplified DNA probed with a plasmid containing the *Bam*HI K region of EBV, demonstrating that a common strain of EBV is shared by patients T and P and the organ donor. (A) Comparison of IR3 amplified DNA. Lanes (from left to right): IB4, 10^{-3}, 10^{-4}, and 10^{-5} μg of LCL DNA; BamHI K, *Bam*HI plasmid DNA (4×10^3 copies); P, 10^{-3} and 10^{-4} μg of patient P pooled LCL DNA; T, 10^{-3} and 10^{-4} μg of patient T LCL (T11) DNA; P3HR1-K, 10^{-5} and 10^{-6} μg of LCL DNA; AG876, 10^{-5} and 10^{-6} μg of LCL DNA. (B) Comparison of donor and patient T IR3 DNA. Donor DNA (DONOR) was extracted from approximately 5×10^6 donor spleen cells. One-fiftieth of the recovered DNA, equivalent to approximately 10^5 cells, was amplified as described in the text and compared with amplified DNA obtained from increasing starting amounts (left to right: 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} μg) of patient T LCL DNA (T): BJAB, 10^{-1} μg of LCL DNA. Numbers at right indicate kilobases.

(data not shown). These results suggest that viral transmission was not via a shared oral route after transplantation, and they further suggest that two relatively independent EBV reservoirs are present, the PBL and the oropharynx. This observation is consistent with recent evidence supporting the hypothesis that bone marrow may be the primary reservoir for EBV (6, 7, 20, 21).

On the basis of the identity established by RFLP analysis, the most probable source of the transmitted virus strain present in both patients appeared to be the donor organs. We obtained a small number of spleen cells from the donor tissue. Since we could expect only 1 of every 10^6 cells to contain virus (17), thus making the virus undetectable through direct Southern blotting, we opted to amplify one of the previously identified regions of polymorphism, IR3, by polymerase chain reaction (PCR) to provide additional support for the mode of transmission. We selected a set of primers, 5'CCAAGTTGCATTGGCTGCAAAGG3' and 5'GGCTCTTTCACGTCCTCTAC3', representing the direct sequence bp 108178 to 108200 and inverted complementary sequence bp 109013 to 109032, respectively, according to the sequence published by Baer et al. (1); these primers were complementary to unique sequences flanking IR3 of EBV. Reactions were carried out in 50 mM KCl–10 mM Tris hydrochloride (pH 8.3)–1.5 mM $MgCl_2$–0.01% (wt/vol) gelatin–200 μM dATP–200 μM dCTP–200 μM dTTP–50 μM dGTP (Perkin-Elmer-Cetus)–150 μM 7-deaza-dGTP (Boehringer) by using 1 μM of each primer (synthesized by the Biological Sciences core facility, University of Pittsburgh) and 1 U of *Taq* polymerase (Perkin-Elmer-Cetus) in a final volume of 25 μl. Samples were overlaid with 30 μl of mineral oil and underwent an initial cycle of 1 min at 96°C, 2 min at 55°C, and 3 min at 72°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Routine amplification did not give rise to detectable DNA when visualized by ethidium bromide staining of agarose gels, so Southern blot analysis of the PCR mixture was carried out. Figure 3 depicts representative autoradiograms of Southern-blotted IR3 DNA amplified by PCR and probed with a plasmid containing the *Bam*HI K region of the viral genome. In this series of experiments, in addition to a negative control (BJAB), a number of positive controls (*Bam*HI K plasmid DNA, P3HR1-K, IB4, and AG876) were run in parallel to confirm the specificity of the reaction and to demonstrate the diversity of repeat units present in IR3 (Fig. 3A). Note that the sequence-predicted 855-bp IR3 PCR product of IB4 comigrates with that of the B95.8-derived *Bam*HI K-containing plasmid; note also that each strain tested produced a PCR product of unique size, ranging from approximately 700 to 1,200 bp, which was distinguishable from the others. The donor's strain of EBV produced a PCR product approximately 700 bp in size which comigrated with the IR3 product representing patient T's strain and one of the two IR3 products representing the two strains of EBV previously characterized in patient P's cells (Fig. 3B). Considering the diversity observed in the IR3 PCR products, the most probable explanation for this observation is that a single strain of EBV was transmitted to both patients via the donor's organs.

Theoretically, the donor's strain of EBV could be main-

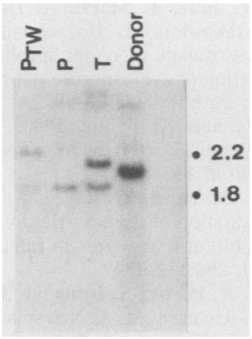

FIG. 4. Southern blot analysis of 10 μg of cellular DNA obtained from LCL derived from patients T and P, demonstrating that proliferation of donor B lymphocytes is not the cause of PTLD. The cellular DNA was digested with *Hin*fI and probed with the minisatellite 33.6 probe. Lanes: Donor, LCL derived from superinfection of the donor spleen cells with B95.8; T and P, pooled LCL derived from the peripheral blood of patients T and P, respectively; P_{TW}, LCL derived from the infection of cord blood lymphocytes with patient P's throat wash. Numbers at right indicate kilobases.

tained within the donor's lymphocytes after transplantation, or the virus could infect the recipient's lymphocytes either by undergoing a lytic infectious cycle or by cell-to-cell transmission. In the first case, we would anticipate that both the transformed PBL and the B cells responsible for the PTLD lesion would be of donor origin. In the latter case, the lymphocytes should be those of the recipient. To determine which is the case, we analyzed the minisatellite DNA patterns of the 15 PBL-derived cell lines, the PTLD lesion-derived cell line of patient T, the PBL-derived cell line of patient P, and the donor LCL (the latter were derived by superinfection of 5×10^6 spleen cells with 100 μl of a filtered supernatant of B95.8 LCL). Representative results that utilize pooled LCL (Fig. 4) demonstrate that the minisatellite DNA pattern differed among all the LCL (the minisatellite DNA probe 33.6 is commercially available and is labeled according to the protocol supplied by the manufacturer [Cambridge Research Biochemicals, Ltd., Cambridge, England]). These results indicated that although the donor's strain of EBV was transmitted to both recipients through the donor's solid organs (heart and lung for patient T, kidney for patient P), lymphoproliferation did not result from the proliferation of donor B cells in either recipient.

In conclusion, RFLP and PCR analyses demonstrate that a single strain of EBV was transmitted from the organs of a single donor to two recipients, both of whom acquired PTLD shortly after transplantation (54 days for patient T, 64 days for patient P). The minor polymorphism we detected within the *Bam*HI B′ region can be attributed to a mutation which occurred during passage in vitro in patient P's cells. This conclusion is based on a previous report of a similar mutation in the *Bam*HI C region of the B95.8-derived umbilical cord blood cell lines IB4 and X50.7 (19). The fact that the EBV strain present in patient P's throat wash-derived LCL is not identical with the strain present in his PBL-derived LCL provides further support for the view that these two viral reservoirs are independent and suggests that the PBL themselves are directly infected at the time of transplantation.

Although the argument that the EBV present in the donor's lymphocytes transform the recipient cells during a second-step transformation event in vitro could be made, it is unlikely in view of the presence of clonal EBV episomal and B-cell populations in early-passage PBL and PTLD lesion-derived LCL from both patients (data not shown) (16). As for the source of virus in the donor organ, it is possible that a sufficient number of EBV-immortalized B lymphocytes remain within the donor tissue, are transplanted with the organ, enter the peripheral circulation, undergo lytic replication, and infect neighboring recipient B lymphocytes. However, given the number of transforming B cells present within a normal individual's peripheral blood, estimated to be fewer than 1 of every 10^6 B lymphocytes (17), and the number of B lymphocytes present in a donor organ, we consider this route unlikely. The rapid infection and proliferation of the donor virus in both recipients introduce the possibility that a more permissive cell type which enables virus to infect the circulating B lymphocytes of the recipients is present within the organ tissue. Since the PTLD observed in both recipients was present in, and thus may arise within, the transplanted organ, it is possible that the recipient's B lymphocytes which infiltrate the organ become infected and proliferate within the organ itself and/or eventually migrate to other areas of the body.

Our demonstration that EBV may be transmitted from donor tissues adds to the growing list of viruses, including other members of the human herpesvirus family (cytomegalovirus [11] and herpes simplex virus [4]) and human immunodeficiency virus (5), which may be transmitted in this manner.

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