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Study of Mother-to-Child Epstein-Barr Virus Transmission by Means of Nested PCRs

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The aim of this study was to investigate the possibility of vertical Epstein-Barr virus (EBV) transmission. We developed two nested-PCR methods for amplifying distinct regions of EBV DNA (BNRF1 and *Bam*HI W) in circulating lymphocytes. Nested PCR was applied to samples obtained from 67 mother-infant pairs within 1 week of birth. We also tested samples from 16 neonates born to human immunodeficiency virus (HIV)-infected mothers to determine whether HIV increases the potential risk of vertical EBV transmission. About half of the 67 women in the first population were positive by nested PCR. Two neonates born to EBV PCR-positive women were also PCR positive. One of the 16 neonates born to HIV-infected women was PCR positive for EBV. These results strongly support the possibility of EBV transmission in utero or during delivery but do not suggest that HIV infection increases this risk. Further studies are required to confirm these findings, to identify the precise mode of vertical EBV transmission, and to determine the outcome for infants who are positive at birth for EBV DNA by nested PCR.

Epstein-Barr virus (EBV) is a member of the herpesvirus family, and serological evidence of infection in more than 90% of the population worldwide is found. In contrast to other herpesviruses, EBV causes no clinical manifestations in the vast majority of individuals. However, when it occurs during adolescence or adulthood, EBV infection can cause a benign lymphoproliferative disease known as infectious mononucleosis.

Following primary infection, EBV latently infects B lymphocytes and persists for life. Like infections with all members of the herpesvirus family, EBV infection can reactivate. During these periodic episodes the virus is shed from the oropharynx and, occasionally, from the uterine cervix (31, 35, 39, 43).

Some herpesviruses, e.g., cytomegalovirus, herpes simplex virus, and varicella-zoster virus, have been associated with congenital or perinatal disease. Cytomegalovirus is considered the virus most commonly transmitted in utero (17, 34, 41). Herpes simplex virus is transmitted predominantly during passage through the birth canal (13, 32, 42). Varicella in pregnancy is poorly characterized; congenital malformations have been described, but the most common problem is maternal varicella in the peripartum period (5, 33, 42). Little work has been done on the outcome of fetal infection by EBV, although several authors have attempted to link congenital malformations to primary maternal EBV infection (4, 15, 22, 25, 38).

Previous attempts to detect EBV transmission relied mainly upon detection of immortalized EBV-positive B-cell lines grown from peripheral blood (6, 45). However, Rickinson and collaborators demonstrated that this outgrowth occurred predominantly by an indirect route involving virus release and reinfection in culture (37). As a consequence, this method cannot detect cells that do not release the virus in culture. Nested PCR offered an alternative approach enabling the detection of very small amounts of EBV DNA, independently of virus production. We investigated putative mother-to-child EBV transmission by developing two nested-PCR-based assays for the detection of EBV genomic DNA in peripheral blood mononuclear cells. As nested PCR is exquisitely sensitive, we chose to amplify two independent regions of the EBV genome to avoid false-positive results. Transmission was first studied in a cohort of 67 mother-neonate pairs. As human immunodeficiency virus (HIV)-infected patients are known to have an increase in several EBV reactivation markers (20, 26, 27, 36, 44), we then studied samples from 16 neonates born to HIV-seropositive mothers.

MATERIALS AND METHODS

Mothers and newborns. Sixty-seven pregnant women attending the obstetrical unit of Saint-Antoine Hospital (Paris, France) between 25 January 1995 and 25 April 1995 were enrolled. From 1 to 7 days postpartum, blood for mononuclear cell isolation was drawn from neonates (1 to 3 ml) and mothers (10 to 15 ml) into EDTA-coated tubes. An additional 5 ml was taken from the mothers for EBV serological analysis. The mothers gave their informed consent.

Children born to women with HIV-1 infection. Mononuclear cells were obtained from 16 neonates less than 1 week old born to HIV type 1 (HIV-1)-infected women attending Paris hospitals between 7 December 1992 and 7 June 1994. This population was chosen because several markers of EBV reactivation are found in HIV-infected patients, which potentially increases the risk of EBV transmission to the infant.

The EBV serological status of the mothers was determined. No stored samples of maternal mononuclear cells were available.

Control subjects. Mononuclear cell samples were obtained from 53 nonpregnant premenopausal HIV-seronegative women.

Cell lines. Namalwa is an EBV-positive B-cell line containing two integrated viral genomes (23). DG75 is an EBV-negative B-cell line (2).

Specific serologic testing for EBV. Immunoglobulin G antibodies to EBV capsid antigen were titrated with an enzyme-linked immunosorbent assay kit (Clark Laboratories, Jamestown, N.Y.).

Nested-PCR assays. Peripheral blood mononuclear cells obtained by Ficoll-Hypaque centrifugation were divided into pellets containing about 10^6 cells and stored at −80°C. For PCR analysis each pellet was resuspended in 100 μl of PCR lysis buffer containing 100 mM KCl, 100 mM Tris-HCl (pH 8), 2.5 mM $MgCl_2$, 1% Nonidet P-40, and 1% Tween 20 and submitted to proteinase K (20 μg) digestion for 4 h at 56°C. Proteinase K was then inactivated by incubation for 10 min at 94°C.

Two nested-PCR methods were developed with primers in independent regions of the EBV genome (BNRF1 and *Bam*HI W domains).

(i) First nested PCR. All samples were tested for EBV DNA by PCR with two sets of nested primers specific for BNRF1 EBV late genes. The first round of amplification (PCR1), with primers C3 (GCATCTACACCTGGATCCGC) and C4 (GGAGACAGAGGGCCACCACG), yielded a 171-bp product. Second-

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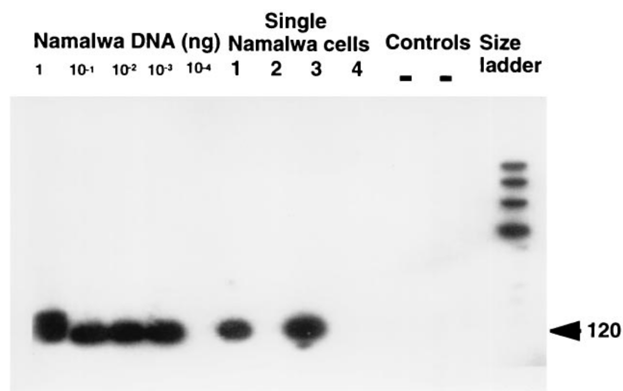

FIG. 1. Assessment of the sensitivity of nested PCR (BNRF1 probe) by Southern blot analysis. Lanes: 1 to 5, serial dilutions of DNA extracted from Namalwa cells. EBV DNA was detected to a dilution of 10^{-3} ng. Lanes: 6 to 9, one Namalwa cell, containing two copies of EBV and isolated by micromanipulation, mixed with 10^5 DG75 cells not containing the EBV genome. EBV DNA was detected in about 50% of samples. Lane 10, negative control (1 ng of DNA extracted from DG75 cells). Lane 11, negative control (10^5 DG75 cells). The arrowhead indicates the 120-bp product.

round PCR (PCR2) was performed with primers C1 (GACAACTCGGCCGTG ATGGA) and C2 (TGAAGTTGGAGGCGGACGAG), yielding a 120-bp product.

First, 1/10 of the lysate (10 μl) was subjected to 40 cycles of amplification (PCR1) in a 50-μl reaction mixture containing 10 pmol each of primers C3 and C4, 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 200 μM each nucleoside triphosphate (NTP), and 0.5 U of *Taq* polymerase. Each amplification cycle included denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 30 s) and was performed in an automated thermal cycler (Perkin Elmer 9600; Cetus). Second, 1/10 of the first reaction mixture was subjected to 30 cycles of amplification (PCR2) in the presence of a mixture containing 10 pmol each of primers C1 and C2, 50 mM KCl, 10 mM Tris-HCl (pH 8), 1 mM $MgCl_2$, 200 μM each NTP, and 0.5 U of *Taq* polymerase. The cycles were identical in PCR1 and PCR2. Each cell extract was submitted to *ras* gene amplification to check for the absence of reaction inhibitors.

PCR products were subjected to electrophoresis on a 2% agarose gel or a 12% acrylamide gel and transferred to a nylon membrane (Boehringer Mannheim).

Southern blotting was performed with an internal digoxigenin-tailed oligonucleotide (TGGCCTGGGCGTGAAGCTGACCTTTGGCTCGGCCTGCT) according to the manufacturer's recommendations (Boehringer Mannheim).

(ii) Second nested PCR. All samples from neonates were tested for EBV DNA by PCR with two sets of nested primers specific for the *Bam*HI W EBV repeat domain. The first round of amplification (PCR′1), with primers B1 (GGAGTG GGCTTGTTTGTGAC) and B2 (TGGGTGTGGTGGAGTGTTGG), yielded a 460-bp product. Second-round PCR (PCR′2) was performed with primers B3 (GCTATTTCTGGTCGCATC) and B4 (CCTTCACTTCGGTCTCCC), yielding a 208-bp product.

One-tenth of the lysate (10 μl) was subjected to 40 cycles of amplification (PCR′1) in a 50-μl reaction mixture containing 10 pmol each of primers B1 and B2, 40 mM KCl, 10 mM Tris-HCl (pH 9.5), 0.5 mM $MgCl_2$, 200 μM each NTP and 0.5 U of *Taq* polymerase. Each amplification cycle consisted of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 30 s) and was performed in the same automated thermal cycler. Then, 5 μl of the first reaction mixture was subjected to 30 cycles of amplification (PCR′2) in the presence of a mixture containing 10 pmol each of primers B3 and B4, 50 mM KCl, 10 mM Tris-HCl (pH 9.5), 1 mM $MgCl_2$, 200 μM each NTP, and 0.5 U of *Taq* polymerase. Each amplification cycle consisted of denaturation (94°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 30 s). Each cell extract was submitted to *ras* gene amplification to check for the absence of reaction inhibitors.

PCR′ products were subjected to electrophoresis on a 1.5% agarose gel or a 12% acrylamide gel and transferred to a nylon membrane (Boehringer Mannheim). Southern blotting was performed with an internal digoxigenin-tailed oligonucleotide (CCCTTCCCTCCCTCGTCTTGCCCTGCGCCC) according to the manufacturer's recommendations (Boehringer, Mannheim).

(iii) Controls. Negative controls consisting of a reaction mixture alone or a DG75 cell lysate were submitted to nested PCR. Phenol-chloroform-extracted Namalwa cell DNA (10 ng per reaction) was used as a positive control.

The sensitivity of EBV-directed nested PCR was first evaluated with phenol-extracted Namalwa cell DNA after serial dilution in lysis buffer. Subsequently, single Namalwa cells were isolated by micromanipulation, mixed with 10^5 DG75 cells, suspended in lysis buffer, and subjected to nested PCRs as described above.

(iv) Prevention of contamination. The usual precautions were taken, including extraction under a laminar flow hood, carrying out each step in a separate room, and use of disposable face masks, gowns, gloves, pipettes, and tips. Controls run in each experiment consisted of a DG75 cell extract, DNA-free PCR mixture, and lysis solution.

Statistical analysis. Data were analyzed by the chi-square test for maternal data and Fisher's test for neonatal data.

RESULTS

Nested PCR. Each amplification reaction was optimized with regard to the $MgCl_2$ concentration and pH; Namalwa and DG75 cell phenol-extracted DNAs were used as positive and negative controls, respectively.

The sensitivity of each procedure was first evaluated on serial dilutions of purified Namalwa cell DNA. The two techniques were specific and highly sensitive, as we detected 1 to 10 copies of viral DNA after agarose gel electrophoresis and Southern blotting (Fig. 1). Second, single Namalwa cells (two viral copies per cell) were isolated by micromanipulation, mixed with 10^5 EBV-negative cells (DG75), lysed, and subjected to nested PCR. Both procedures detected two viral copies (Fig. 1), and the PCRs had similar sensitivities (data not shown). EBV genome detection was also specific, as no amplification product was observed after agarose gel electrophoresis or Southern blotting when purified DNA from varicella-zoster virus, cytomegalovirus, and herpes simplex virus types 1 and 2 were tested (data not shown).

EBV genome detection in mothers and neonates. All mothers were positive for immunoglobulin G antibodies to EBV capsid antigen.

By the first nested PCR, EBV DNA was detected in 30 of 53 nonpregnant and 36 of 67 pregnant women, by both agarose gel electrophoresis and Southern blot analysis (Table 1), which is in agreement with the low frequency of EBV-infected B cells in healthy seropositive carriers (30). There was no significant difference between the two groups ($P = 0.75$, chi-square tests), suggesting that pregnancy does not induce any significant increase in the amount of infected B cells. Five newborns of 83 were positive according to the first nested PCR (3 of 67 neonates born to HIV-1-seronegative mothers and 2 of 16 neonates born to HIV-1-seropositive mothers). Note that in two neonates (one from each cohort) EBV DNA was detected only once (in two repeats) by the first nested PCR and could not be detected by the second nested PCR, suggesting possible contamination. The three other newborns were repeatedly positive by both nested PCRs, which rules out contamination (Fig. 2 and 3). Thus, except for results for these two neonates, the two nested PCRs provided concordant results on two independent repeats. The mothers of EBV PCR-positive neonates were

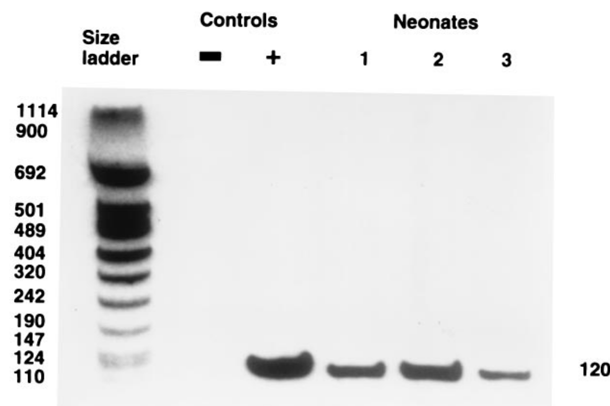

FIG. 2. Southern blot analysis of nested-PCR-positive samples with the BNRF1 probe. Numbers on the sides of the gel are molecular sizes, in base pairs.

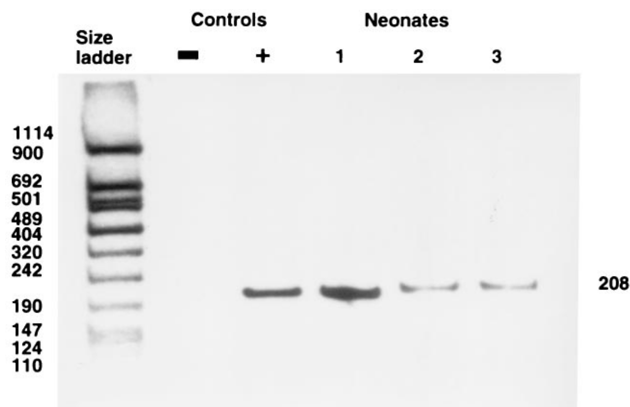

FIG. 3. Southern blot analysis of nested-PCR-positive samples with the *Bam*HI W probe. Numbers on the sides of the gel are molecular sizes, in base pairs.

repeatedly positive for EBV, but we found no correlation between EBV detection in mothers and neonates, possibly because of the small number of positive samples. Similarly, there was no significant effect of EBV transmission rate for HIV-seropositive and HIV-seronegative mothers ($P = 0.48$, Fisher's test). Each sample was positive for *ras* amplification, eliminating the presence of a PCR inhibitor in the negative samples. None of the EBV-negative (DG75) cell samples extracted in parallel with the study samples yielded amplification products.

DISCUSSION

Primary maternal EBV infection is rare. Only 1.3 to 4.2% of pregnant women are EBV seronegative in industrialized countries (11, 14, 19, 24), which explains the low rate of seroconversion among pregnant women in the various published studies: 2 of 102 (7), 3 of 250 (1), 1 of 1,729 (24), 35 of 1,939 (12), and 6 of 2,752 (18). Cases of birth defects, prematurity, and low birth weight were observed but could not be linked to EBV infection. Isolated cases of birth defects after maternal primary EBV infection have also been reported (4, 14, 25, 38), but no samples were obtained from the infants. Mother-to-child EBV transmission was suggested but not proven.

Latent maternal EBV infection can also reactivate during pregnancy: Fleisher and Bolognese (10) reported detecting anti-early antigen antibodies in 55% of a group of pregnant women, compared with 22 to 32% of nonpregnant women, and Costa et al. (7) reported prevalences of 37.7 and 23% for pregnant and nonpregnant women, respectively. Similarly, we have found that 13% of pregnant women have antibodies to the *Bam*HI EBV replication activator), compared with 3% of nonpregnant women (29). However, the consequences of such reactivation for the infant remain to be determined. One study showed a correlation between the presence of anti-early antigen antibodies during pregnancy and fetal defects (18), but this was not confirmed in another study (10).

Several authors have tried to identify fetal EBV infection by testing umbilical cord blood and neonatal oropharyngeal secretions for EBV-transformed lymphocytes. In a study performed in 1973, 1 of the 696 specimens tested was positive (6); EBV-infected cells were present in both the child's blood and oropharynx, and the infant was apparently healthy for at least 24 months. A second study (45) identified EBV-transformed cells in 1 of 40 specimens of umbilical cord blood. Visintine et al. detected EBV in a premature child 16 days after birth. The authors also reported the case of an infant born to a woman who developed infectious mononucleosis late in pregnancy; the infant had psychomotor retardation and cortical blindness at 4 months of age, but EBV infection could not be proven. Finally, Joncas and his group (21) reported that no EBV-transformed cells could be isolated from peripheral blood in a group of 137 neonates. In short, it has not been proven that congenital birth defects can be associated with fetal EBV infection or that mother-to-child EBV transmission can occur, possibly because the procedures used were not sensitive enough.

We describe nested-PCR-based assays sensitive enough to detect a single EBV-infected cell. These procedures were applied to the detection of EBV DNA in peripheral cells of neonates born to HIV-1-seronegative and HIV-1-seropositive women. EBV DNA was detected (Table 1) as frequently in pregnant women as in nonpregnant women. This prevalence is similar to that in Gopal's group of healthy adults (16) and suggests that there is no significant effect of pregnancy on the percentage of infected peripheral blood mononuclear cells, even though previous work has indicated an increase in several serological markers of reactivation (antibodies to early antigen and the *Bam*HI EBV replication activator) during pregnancy (7, 10, 18, 29).

To assess EBV transmission to neonates, we ruled out cord blood analysis (because of contamination by infected maternal lymphocytes) and chose to sample neonatal peripheral cells between 1 and 7 days after birth. Infection during passage through the birth canal seems unlikely, despite the potential presence of EBV in cervical secretions (39), as one infected infant was delivered by cesarean section. In addition, samples were always obtained before day 4 of life, which is too early to detect the EBV genome in the neonates' peripheral lymphocytes. Nested PCR was positive for 2 of 67 neonates and for 1 of 16 neonates born to HIV-1-seropositive mothers, suggesting that mother-to-child transmission of free EBV or maternal EBV-infected cells can occur during pregnancy in nonprimary infection.

EBV positivity might have resulted from detection of infected maternal cells present in cord blood, as the frequency of EBV detection in neonates (3 in 83) was close to that of maternal cell transmission (1 in 47 cases in the study of Socié et al. [40]). However, this possibility is unlikely. In our experiments, 10^5 cells were used for PCR. Assuming that maternal cells account for 1% of cord blood lymphocytes (40), the sample would have contained at most 1,000 maternal cells. As B cells accounted for about 30% of total lymphocytes, this would mean that the sample contained roughly 300 maternal B cells. Miyashita and collaborators (30) recently reported that the frequency of infected B cells varied from 23 to 625 per 10^7 B cells. This makes the possibility of detecting EBV-infected cells of maternal origin in a neonate very unlikely. However, our data are still consistent with the assumption that infected maternal B cells are a vehicle for transmission, but this probably implies replication of the virus and subsequent infection of neonatal B cells. In addition, transmission of free EBV is

TABLE 1. EBV DNA amplification

Subject group (*n*)	No. of patients (%) infected as determined by nested PCR	
	First	Second
Nonpregnant women (53)	30 (56.6)	
Pregnant women (67)	36 (53.7)	
Children of HIV-seronegative women (67)	3	2
Children of HIV-infected women (16)	2	1

unlikely, as the EBV genome is rarely detectable in the sera of healthy EBV carriers (46). Maternal antibodies may play a protective effect, as suggested by several epidemiological studies. Indeed, Biggar and collaborators (3) demonstrated that specific maternal anti-EBV antibodies protected infants up to the age of 6 months. However, it was not possible in our study to monitor the EBV-positive neonates outside the hospital context, and we were thus unable to detect long-term persistence of EBV in these children. Early transmission of EBV can induce tolerance of the viral antigen and consequently can limit the specific immune response, as for early hepatitis B (28). This raises the interesting possibility that early transmission may favor subsequent EBV-associated disorders. Burkitt's lymphoma, which is endemic in East Africa, appears between 7 and 9 years of age, at least in Uganda (9). The primary EBV infection occurs early in this country, with 100% of children being EBV seropositive between 2 and 3 years of age, and seroconversion can occur as early as 3 months of age. There thus appears to be a correlation between early EBV infection and the occurrence of Burkitt's lymphoma in children (8, 9) that calls for further studies on the prevalence of intrauterine EBV transmission in these countries and its possible link with Burkitt's lymphoma.

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M. C. Meyohas and V. Maréchal contributed equally to this work.

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