Direct demonstration of persistent Epstein–Barr virus gene expression in peripheral blood of infected common marmosets and analysis of virus-infected tissues in vivo

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Epstein–Barr virus (EBV) infection in animal model systems has been studied previously in marmosets and tamarins using serology and PCR of saliva. Here we directly demonstrated long-term persistence of EBV in the peripheral blood of marmosets by assaying EBER RNA expression. A new reverse transcription–PCR assay, able to distinguish a naturally occurring strain polymorphism in EBER 2 that may be useful as a strain marker for monitoring persistence and interactions between multiple strains in the same animal or person, has been developed. In situ hybridization and immunohistochemistry have also been used to search for EBV-infected cells in the animals. The carrier state in the common marmoset is similar to that of humans in that it is asymptomatic, long-lived and displays a very low level of circulating virus-infected cells. It differs from the human in lacking the characteristic antibody response to EBNA 1.

Introduction

Progress in Epstein–Barr virus (EBV) genetics (Rickinson & Kieff, 1996; Robertson et al., 1996) and the construction of recombinant EBV strains (Kempkes et al., 1995) have raised the possibility of using EBV-based vectors for transfer of genetic information into humans (Banerjee et al., 1995). The consistent presence of EBV in several different types of tumour has also led to the proposal of strategies to target therapeutic agents to the tumour cells (Gutierrez et al., 1996; Franken et al., 1996; Evans et al., 1997). Testing such novel therapeutic agents and analysis of their persistent effects in the human host is likely to require preliminary analysis in an animal model system. EBV infection is restricted to primate species but cross-reaction of some EBV markers with those of endogenous herpesviruses of Old World monkeys has meant that the common marmoset (Callithrix jacchus) and the cotton-top tamarin (Saguinus oedipus), which do not appear to have herpesviruses that cross-react significantly, have been the most useful species in this regard. Of these two species, the common marmoset is much more readily available for research as a result of established breeding colonies.

The cotton-top tamarin was found to be particularly sensitive to the tumorigenic effects of EBV and, although a recent study has found EBV-infected lymphoid cells in immunized cotton-top tamarins protected against the development of EBV-driven lymphoma, tumour formation complicates its use as a model of human EBV infection (Niedobitek et al., 1994). In contrast, EBV infection of the common marmoset only very rarely results in spontaneous tumour formation and has been discussed as a model for persistent EBV infection of humans (Wedderburn et al., 1984; Cox et al., 1996). Long-term virus persistence has previously been inferred from serology and PCR of virus DNA in saliva (Cox et al., 1996), but virus has not been directly demonstrated in the blood. Current models of EBV persistence in humans consider that the virus persists in blood cells in the B lymphocyte lineage (Niedobitek et al., 1992; Niedobitek & Young, 1994; Tierney et al., 1994; Miyashita et al., 1995; Khan et al., 1996).

In this paper, we directly demonstrate EBV gene expression in the peripheral blood of infected common marmosets. In one animal, this was 10 years after the primary infection with EBV.
We also further analyse the serology of the infected animals and characterize a polymorphism in an EBV gene whose expression can be detected in peripheral blood samples, so that this could be used as a marker of EBV strains in virus co-infection or superinfection experiments. In the human, EBV is thought to persistently infect a subpopulation of B lymphocytes (Yao et al., 1985; Niedobitek et al., 1992; Tierney et al., 1994; Miyashita et al., 1995; Decker et al., 1996; Khan et al., 1996) and perhaps also to undergo replication in epithelial cells in the oropharynx. By dissecting major tissues of marmosets with persistent infection and in situ hybridization for EBER RNA, the pattern of infection in these animals has been studied directly.

**Methods**

**Animals.** Common marmosets were kept and infected with M81 EBV (Desgranges et al., 1976) by injection into Waldeyer’s ring as described previously (Wedderburn et al., 1984; Cox et al., 1990; Mackett et al., 1996). Marmoset 160 (male) was infected with M81 EBV at age 1 month and blood was taken for reverse transcription (RT)–PCR and serology 10 years later. Animals 105S (male) and 105F (female) were twins born to EBV-infected parents. They were infected orally at age 3 months and blood was taken for RT–PCR and serology 8 months later. Marmosets 160, 105S and 105F were killed and dissected for in situ hybridization and immunohistochemical analysis 8 months after the blood was taken for RT–PCR and serology. Marmosets 843 and 938 were from a separate breeding colony and had no history of EBV infection.

Cotton-top tamarins were kept and infected by intravenous inoculation as described previously (Niedobitek et al., 1994); a sub-tumorigenic dose of EBV was used. Animal 212 was not infected with EBV. Animal 152 was infected with B95-8 EBV, recovered from EBV-induced disease, and was then further inoculated with EBV. Blood was taken for RT–PCR 6 months after the final inoculation with EBV.

**Cell purification and RNA extraction.** Marmosets were anaesthetized by intramuscular injection in the thigh with 0.07 ml ketamine (Vetalar; 100 mg/ml) using a 25G needle. After about 10 min, when the animals were very placid, blood (0.5–2.0 ml) was withdrawn from veins in the thigh using a 25G needle. For preparation of leukocytes, the blood (Vetalar; 100 mg)

| **Results** |

**EBER RNA expression in the peripheral blood of infected marmosets**

Leukocytes were prepared from heparin-treated peripheral blood samples by centrifugation of the diluted blood on Ficoll paque. RNA was extracted from the cells and cDNA was a mixture containing 25 mM each of dATP, dGTP, dCTP and dTTP, and 1 µl Moloney murine leukaemia virus reverse transcriptase were added. The reaction mixture was incubated at 37 °C for 1 h, heated at 65 °C for 5 min and then stored at −70 °C.

**Primers for PCR, blotting and probing for EBER 1, QUK, Y3UK, LMP1 and LMP2A have been described previously (Tierney et al., 1994).** Primers for EBER 1 were P0364 (AAAAACATCCCCAGCACCGCC) and P0365; conditions were 92 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min for 40 cycles. The product (5 µl) was electrophoresed on a 2% agarose gel, blotted to nitrocellulose or nylon (nylon was found to bind the very small PCR products better) and probed with 32P-labelled P0366 (ACGGTGTCTGTTGGTGTCTT). PCR for EBER 2 used P4112 (TGC-GTATGTTTTCGGA) and P4114; conditions were 92 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min for 40 cycles.

**Western blots.** For serum, non-heparinized blood was allowed to clot overnight at room temperature; the clear serum was removed and microfuged for 2 min to remove insoluble material. Protein extracts were made from cell lines by collecting cells from 10 ml culture by centrifugation, dissolving the pellet in 0.5 ml SDS gel sample buffer, sonication to disperse the DNA and heating to 90 °C for 2 min. Aliquots were electrophoresed on 12.5% SDS gels and the gels were blotted to nitrocellulose. After blocking with 1% dried milk in TBST (150 mM NaCl; 10 mM Tris–HCl, pH 7.5; 0.05% Tween 20), the sera were applied at a dilution of 1:100 in TBST. After incubation overnight at 4 °C, the filters were washed with TBST and then treated with 125I-labelled protein A at (1:1000) for 5 h, further washed with TBST and autoradiographed.

**In situ hybridization.** Plasmids pBSJJ1 and pBSJJ2, containing inserts specific for EBER 1 and EBER 2, were used to generate 32P-labelled RNA probes by in vitro transcription as described by Niedobitek et al. (1991). For in situ hybridization, the antisense probes derived from these plasmids were mixed to increase sensitivity. Similarly, the sense control probes were also applied as a mixture. Paraffin sections were subjected to in situ hybridization as described in detail previously (Niedobitek et al., 1994). Following hybridization overnight at 50 °C, sections were extensively washed and then dipped into IIford G5 photographic emulsion. The slides were exposed for 3–10 days, developed, fixed and counterstained with haematoxylin.

**Immunohistochemistry.** All tissues were analysed with BZ1 (Young et al., 1991), which recognizes the BZLF1 protein of EBV. Tissues containing EBER-positive cells were also subjected to immunohistochemistry with monoclonal antibody CS1-4 (Rowe et al., 1987), specific for LMP1, and monoclonal antibody PE2 (Young et al., 1989), which detects EBNA 2. These antibodies were obtained from Dako. Sections from the tongue were also stained using monoclonal antibody EADE31 (Novocastra), which is specific for the diffuse component of the EBV-encoded early antigen (EA-D).

Before incubation with the primary antibodies, paraffin sections were dewaxed and subjected to antigen retrieval by microwave irradiation as described by Niedobitek et al. (1994). Bound primary antibodies were then detected using the alkaline phosphatase anti-alkaline phosphatase method as described by Niedobitek et al. (1994).
Epstein–Barr virus persistence in marmosets

Fig. 1. Diagram of PCR primers used for amplification and detection of EBER 1 and EBER 2, shown beneath a scale in kb corresponding to the standard numbering of the B95-8 EBV genome (Baer et al., 1984).

Fig. 2. Blot of EBER 1 RT–PCR of marmoset blood samples. Randomly primed cDNA synthesized from peripheral blood leukocyte RNA from the indicated animals or from B95-8 cells was PCR-amplified for EBER 1. The products were electrophoresed, blotted and probed with a radioactively labelled EBER 1 oligonucleotide (P0366). The negative controls for the PCR were cDNA control (reverse transcriptase omitted from the reverse transcription) and PCR control (cDNA omitted from the PCR).

Fig. 3. Sensitivity of the PCR assay of EBER 1 and EBER 2. Decreasing amounts of B95-8 cell genomic DNA template were used in standard PCR reactions for EBER 1 or EBER 2. Tracks W are water controls for PCR and track M is the DNA size markers (a HaeIII digest of φX174 DNA). DNA template amounts were 100 ng, 20 ng, 4 ng, 0.8 ng or 0.16 ng per 50 µl PCR reaction; 5 µl samples were loaded on the gel.

synthesized using random primers; samples of this were used for PCR analysis. Using primers (Fig. 1) and a Southern blotting assay that have been developed for the analysis of EBER 1 in human blood samples (Tierney et al., 1994), EBER 1 expression could be detected (Fig. 2) in the cDNA made from peripheral blood of an infected common marmoset (160) and an infected cotton-top tamarin (B152) but not in cDNA from uninfected marmosets (843 and 938) from a separate colony or an uninfected cotton-top tamarin (B212). Similar PCR experiments using primer pairs (Tierney et al., 1994) for the QUK, Y3UK, LMP1 and LMP2A transcripts failed to detect any signal in the cDNA from 160 and B152 (data not shown). EBER RNA is expressed at a much higher level than virus mRNAs so this lack of detection of mRNAs may have resulted from insufficient sensitivity of these assays. Attempts to directly PCR amplify the virus DNA also failed, probably for the same reason. Because of the small samples of blood that can be taken from the marmoset, very sensitive assays are required to detect virus gene expression.

If the marmoset is a valid model for persistent EBV infection, the animals may be useful in the future to study consequences of superinfection with a genetically marked strain of EBV in an animal that already carries EBV (comparable to an already infected human encountering a new strain of EBV). We therefore sought to establish an assay for EBV infection that could be used on peripheral blood but could distinguish between EBV strains. EBER 1 and EBER 2 have been sequenced from six EBV strains (Arrand et al., 1989). EBER 1 was found to be invariant but a point change in EBER 2 has been described in some strains; this polymorphism happens to introduce a restriction site for Sau3A. EBV strains of the B95-8 or M81 type lack the Sau3A site but strains of the M-ABA type contain this site. RT–PCR for EBER 2 has not been described previously so we tested various combinations of primers predicted from the B95-8 EBV sequence (Baer et al., 1984) under a variety of conditions before defining primers which could amplify EBER 2. When tested on dilutions of B95-8 EBV DNA, the PCR conditions for EBER 2 were at least as sensitive as those for EBER 1 (Fig. 3). By specifically priming
Fig. 4. EBER 2 PCR of marmoset blood samples. Equivalent amounts of cDNA from the indicated sources were analysed by PCR for EBER 2 using primers P4112 and P4114. The cDNA control was a negative control taken through the entire reaction but with RNA omitted from the cDNA synthesis. Markers are a HaeIII digest of φX174 DNA.

Fig. 5. Sau3A digest of labelled PCR products. 32P-Labelled EBER 2 PCR products were digested with Sau3A as indicated and electrophoresed on a polyacrylamide gel. Water tracks are a negative control for the PCR, in which water was substituted for the cDNA.

Fig. 6. Western blots using marmoset sera. Sera from the indicated animals or a normal human EBV carrier (PF) were used to probe Western blots of protein extracts of Akata cells. Tracks: 1, Akata clone that has lost its EBV; 2, EBV-positive Akata; 3, EBV-positive Akata induced into the lytic cycle by anti-Ig treatment. The positions at which EBNA 1 and an abundant group of EBV lytic cycle antigens (bracketed in the 160 panel) migrate are indicated.
cDNA synthesis with an EBER 2 primer and then performing PCR for EBER 2, it was possible to greatly increase the specificity of detection of EBER 2 in the RNA from marmoset peripheral blood samples, so that a single major PCR product was obtained and no Southern blotting was necessary to detect EBER 2 gene expression. Using this assay, EBER 2 gene expression was demonstrated in the peripheral blood of marmosets 160 and 1057 but not in the blood of 1056 (Fig. 4). The EBER 2 PCR products were reamplified incorporating a low level of radioactively labelled nucleotide, digested with Sau3A and then analysed by PAGE. Autoradiography readily revealed the distinction between B95-8–M81 type EBER 2 and the M-ABA type (Fig. 5).

To confirm that the EBER 2 PCR product was from EBV and not from an unknown marmoset equivalent virus that might be present, the EBER 2 PCR product from 1057 was cloned and sequenced and found to be identical to the B95-8 EBV sequence (Baer et al., 1984) in this region, confirming the infection with EBV.

**Serology of marmosets infected with EBV**

Antibodies to EBV replicative cycle proteins have been reported previously in these infected animals using immunofluorescence tests but no antibodies were found to EBNA 1 (Wedderburn et al., 1984; Emini et al., 1986; Cox et al., 1996). Antibodies to EBNA 1 are characteristic of long-term persistence of EBV in the normal human carrier. Since the previous serology had used conjugated second antibodies designed for human primary antibodies for the detection of the marmoset antibodies, we applied an alternative method of detection. Western blots of protein extracts from a panel of cell lines that permit identification of relevant EBV proteins were probed with the marmoset sera using 125I-labelled protein A to detect the bound antibodies. Although serum from a normal human carrier of EBV recognized EBNA 1, no evidence of antibodies for EBNA 1 was found in the marmoset sera (Fig. 6). One marmoset serum had high levels of antibodies to EBV replicative cycle proteins (Fig. 6); this animal (160) had previously been reported to have high levels of EBV antibodies as shown by immunofluorescence (Cox et al., 1996). The binding of protein A to these marmoset antibodies indicates that EBNA 1 antibodies would have been detected by this technique had they been present at similar titres to those in the human serum.

**Survey of marmoset tissues for EBV persistence**

The marmosets were killed and dissected. Biopsies from major organs and tissues were either immediately snap-frozen or fixed in formalin. Sections were then analysed for expression of EBER 1–EBER 2. No cells expressing EBER 1–EBER 2 were detected by these methods in sections of 1056 and 1057 (Table 1). In animal 160, a few labelled cells were seen following EBER

### Table 1. EBER in situ hybridization of marmoset tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Marmoset 1056</th>
<th>Marmoset 1057</th>
<th>Marmoset 160</th>
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<tbody>
<tr>
<td>Nasopharynx</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tonsil</td>
<td>+</td>
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<tr>
<td>Base of tongue</td>
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<td>Thymus</td>
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<tr>
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<td>Small intestine</td>
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<tr>
<td>Large intestine</td>
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<td>Liver</td>
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<tr>
<td>Pancreas</td>
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<td>+</td>
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<td>Adrenal gland</td>
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<tr>
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<tr>
<td>Aorta</td>
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<td>Lung</td>
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<td>Kidney</td>
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<td>Urinary bladder</td>
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<tr>
<td>Testis</td>
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<td>Skeletal muscle</td>
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*in situ* hybridization. These cells were found in a para-parotid lymph node and also in the kidney, which contained a marked interstitial lymphoid cell infiltrate (Fig. 7). Interstitial nephritis is frequently present in old marmosets (de The et al., 1980). The morphology of the EBV positive cells was consistent with small lymphocytes and, in the kidney, they were found in association with the interstitial lymphoid cell infiltrate. Expression of the EBV-encoded proteins LMP-I, EBNA 2 and BZLF1 was not detectable at the immunohistochemical level in these tissues. BZLF1 protein expression was additionally tested in all tissues listed in Table 1 but no positive signal was detected.

EBV has been shown to replicate in the human epithelial lesion of the tongue known as oral hairy leukoplakia (Greenspan et al., 1985; Young et al., 1991). Stained sections from the tongue of all three animals were therefore also stained using monoclonal antibody EADE31, which is specific for the diffuse component of the EBV-encoded early antigen (EA-D), associated with viral replication. No specific reactivity was found.
Discussion

Marmosets and tamarins have been studied for many years as model systems for EBV infection but this is the first direct demonstration of the expression of EBV genes in the peripheral blood of infected animals. Furthermore, in the common marmoset, the infection was demonstrated up to 10 years after the infection with EBV, confirming the long-term persistence of infection, as is found in the human (the typical life-span of a common marmoset in laboratory conditions is about 10–12 years). EBV had been demonstrated previously by PCR in the saliva of some of these animals (Cox et al., 1996) but EBV is considered to persist in the B cells of humans and it was therefore important to establish persistence in the blood of the animals. The infection in humans is normally asymptomatic and this was also true in the common marmosets. There are thus some important similarities between the common marmoset infection and the human one.

In these experiments, the virus load detected in the persistently infected common marmoset was found to be extremely low (as in the human). This contrasts with slot-blotting results obtained using DNA extracted from certain tissues of other similarly infected marmosets (Cox et al., 1996). Different techniques with different sensitivities and different animals were used but the results are still surprising. Animal 160 was seropositive for anti-VCA and anti-EA-R for many years and at least three naive females seroconverted soon after being housed with it so it is likely that it produced significant amounts of virus and infected these females. It is conceivable that the previously reported virus DNA reflects a state of virus persistence in which none of the markers of gene expression studied are expressed but there is little evidence for the existence of such a state. The low rate of virus carriage observed in these marmosets contrasts with the cotton-top tamarin, where virus-infected cells were readily detected using the same methods in tissue sections, even in the absence of EBV induced lymphoma (Niedobitek et al., 1994).

Antibodies to EBNA 1 are a characteristic feature of EBV persistence in the human but these were not detected by Western blotting using sera from the infected marmosets.
EBNA 1 antibodies had also not been detected previously using immunofluorescence (Wedderburn et al., 1984). This therefore appears to be a real difference between the marmoset and human persistent infections, although it is interesting to note that some models for persistence in humans envisage a state of EBV infection in which no EBNA 1 is expressed as being the most latent state of EBV infection (Qu & Rowe, 1992). Marmosets are known to express MHC molecules with only a low level of variation (Watkins et al., 1990); combined with the limited genetic diversity present in a closed breeding colony, it is possible that the marmosets lack the appropriate MHC to permit an effective immune response to EBNA 1. Since EBNA 1 contains a sequence that in humans prevents its presentation to the immune system (Levitskaya et al., 1995), it seems most likely that the human EBNA 1 antibodies are produced from fragments of EBNA 1 derived from cells containing EBV that have died and released their contents. The EBNA 1 antibodies are thus not seen as a mechanistic part of EBV persistence so their absence in the marmoset may not devalue the marmoset as a model system for studying EBV infections in vivo.

Antibodies to EBV lytic cycle proteins were confirmed in the serum of marmoset 160. In spite of the presence of antibodies to lytic cycle antigens, none of the wide range of tissues analysed in marmoset 160 showed staining for the BZLF1 lytic cycle protein. It may be noteworthy that the anti-EA serology primarily indicated reactivity with EA-R, which was not measured in our experiments. Animal 160 seems in general to have had a higher virus load than 1056 or 1057 in the sense that it showed evidence of EBV replication in the immune response and was the only animal in which cells containing EBV were confirmed directly by in situ hybridization. It thus remains possible that there is a site of virus replication which we failed to uncover in our survey of tissues or that we examined the correct tissues but the site of replication might be very localized and perhaps was missed in our experiments. It is unclear from our results whether animal 1056 had persistence of EBV in the blood since no signal was detected in the EBER PCR reaction. Virus shedding in the saliva and a low level of EBV lytic cycle antibodies (Cox et al., 1996) have been found with this animal (C. Cox & N. Wedderburn, unpublished results) so it does appear to have had a persistent EBV infection.

The long-term persistence of EBV in humans and well-characterized virus promoters that are able to direct gene expression during persistence suggests that EBV might conceivably be used as part of a hypothetical vector system to permit long-term expression of foreign genes in humans. Since most adult humans are already infected, such an approach would require the ability to infect a person with a second virus strain. To detect a second strain using very small blood samples, as can be obtained from a small animal like a marmoset, a very sensitive strain-dependent assay was developed for EBV. This utilized a sequence polymorphism in the EBER 2 gene; a highly efficient and very specific RT–PCR assay for EBER 2 was established that could distinguish between the polymorphic virus strains. The ability to distinguish between polymorphic strains in vivo would also be useful for analysing the natural history of EBV infection and interplay between strains in vivo. Our results provide further evidence that the common marmoset is a useful model in which persistence of EBV infection can be studied and lay the groundwork for experimental systems that may be used for this in the future.

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References


