Characterization of an Epstein–Barr virus-related gammaherpesvirus from common marmoset (Callithrix jacchus)

Hal B. Jenson,1,2 Yasmin Ench,1 Yanjin Zhang,1 Shou-Jiang Gao,1,2 John R. Arrand3 and Michael Mackett3

1,2 Departments of Pediatrics1 and Microbiology2, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA
3 Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester, UK

A gammaherpesvirus related to Epstein–Barr virus (EBV; Human herpesvirus 4) infects otherwise healthy common marmosets (Callithrix jacchus). Long-term culture of common marmoset peripheral blood lymphocytes resulted in outgrowth of spontaneously immortalized lymphoblastoid cell lines, primarily of B cell lineage. Electron microscopy of cells and supernatants showed herpesvirus particles. There were high rates of serological cross-reactivity to other herpesviruses (68–86%), but with very low geometric mean antibody titres [1:12 to human herpesvirus 6 and 1:14 to Herpesvirus papio (Cercopithecine herpesvirus 12)]. Sequence analysis of the conserved herpesvirus DNA polymerase gene showed that the virus is a member of the lymphocryptovirus subgroup and is most closely related to a lymphocryptovirus from rhesus macaques and is closely related to EBV and Herpesvirus papio. High seroprevalence (79%, with geometric mean antibody titre of 1:110) among 28 common marmosets from two geographically distinct colonies indicated that the virus is likely present in many common marmosets in captivity. A New World primate harbouring a lymphocryptovirus suggests that this subgroup arose much earlier than previously thought.

Introduction

Humans are the only known host for Epstein–Barr virus (EBV; Human herpesvirus 4), a ubiquitous gammaherpesvirus that is the principal cause of infectious mononucleosis and is also associated with several human tumours including Burkitt’s lymphoma, nasopharyngeal carcinoma, B cell lymphoproliferative disorders, Hodgkin’s disease and leiomyosarcomas (Niedobitek et al., 2001). EBV-like viruses in closely related primates have been recognized for over 25 years including Herpesvirus gorilla (Pongine herpesvirus 3) (Neubauer et al., 1979), Herpesvirus pan (Pongine herpesvirus 1) (Gerber et al., 1976; Landon et al., 1968), Herpesvirus pongo (Pongine herpesvirus 2) (Rabin et al., 1978) and Herpesvirus papio (Cercopithecine herpesvirus 12) (Falk et al., 1976, 1977), all belonging to the lymphocryptovirus subgroup of the gammaherpesvirus subfamily. More recently, lymphocryptoviruses have been identified in lymphoid tumours of several macaque species including cynomolgus macaque (Macaca fascicularis) (Hayashi et al., 1995, 1999; Li et al., 1994), stump-tailed macaque (Macaca arctoides) (Wutzler et al., 1995), pig-tailed macaque (Macaca nemestrina) (Rivadeneira et al., 1999) and rhesus macaque (Macaca mulatta) (Moghaddam et al., 1997, 1998; Rangan et al., 1986; Rivailler et al., 2002). Various models for EBV-related lymphoid disease and infection have been developed from these findings. The incidence and prevalence of most of these viruses in wild or captive animals are not known, although the epidemiology of Herpesvirus papio in a large baboon colony is very similar to that of EBV in humans (Jenson et al., 2000). All the host primate species for these lymphocryptoviruses are from the Old World. It has been speculated that the inability to develop authentic animal models for EBV in these species is due to the presence of endogenous infection with species-specific gammaherpes-
viruses and cross-reactive antibodies to EBV (Kalter et al., 1972; Levy et al., 1971). In contrast, New World monkey species, including the cotton-top tamarin (Saguinus oedipus oedipus) (Epstein et al., 1985), owl monkey (Aotus trivirgatus) (Epstein et al., 1973) and common marmoset (Callithrix jacchus), develop B cell lymphoma upon challenge with EBV (Falk et al., 1976).

The common marmoset is a New World monkey of the family Callitrichidae that, under experimental conditions, may be infected with EBV with a humoral response similar to that of humans and unlike the response to EBV infection of the cottontop tamarin or the owl monkey (Wedderburn et al., 1984). Following EBV infection of common marmosets, EBV-associated tumours have been reported in some studies (Falk et al., 1976) but not in others (Ablashi et al., 1978). Several studies suggest the utility of this animal model for the development of EBV vaccines (Cox et al., 1996, 1998; Mackett et al., 1996; Wedderburn et al., 1984), although difficulties have been encountered in the serological assessments of EBV infection.

Previously, gammaherpesviruses were believed to infect only humans and Old World primates. A gammaherpesvirus, Callitrichine herpesvirus 3, closely related to EBV has been identified in common marmosets in association with fatal lymphoproliferative disease (Ramer et al., 1984), although difficulties have been encountered in the serological assessments of EBV infection. Several studies have identified six cell lines from common marmosets that were seronegative for EBV and Herpesvirus papio (animals 57, 184, 244, 11340, 11344, 11345) and one cell line from a common marmoset that had an antibody titre of 1:5 to EBV and Herpesvirus papio (11348).

Methods

- **Cell cultures for spontaneous lymphoblastoid cell lines.** Plasma and peripheral blood mononuclear cells (PBMCs) were obtained from a total of 28 common marmosets (Callithrix jacchus), including 12 animals from Charles River Primates, Inc., Houston, TX, USA, and 16 animals from the Wisconsin Regional Primate Research Center, Madison, WI, USA. All of the animals were adults (> 15 months for common marmosets) from 21–37 months old, approximately 240–260 g in body weight, born and raised in captivity in the respective colonies. PBMCs from 16 animals were separated using Histopaque 1077 (Sigma), washed twice and resuspended with 5 ml RPMI-1640 (Gibco/Invitrogen) supplemented with 20% foetal bovine serum (FBS; HyClone), 1.5% glutamax-1 (Gibco/BRL Life Technologies), 50 µg/ml gentamicin (Sigma), 5 µg/ml Fungizone (Sigma) and 0.2 µg/ml cyclosporin (Sandimmun, Novartis). PBMC from the remaining 12 animals were separated and frozen in cryoprotective medium (above medium with 10% FBS and 10% DMSO) until EBV serologies were performed. Generation of spontaneous cell lines from PBMCs of 24 marmosets was attempted (cells from animals 57, 184, 244 were not tested because these animals were seronegative; animal 11344 was attempted but lost in culture) by incubation at 37 °C with 5% CO₂ with replacement of half of the volume of medium every 3–4 days. Viable cell counts were performed by trypan blue exclusion after 8 weeks in culture and then periodically while cells remained viable. Cultures with no viable cells at 10 weeks were discarded. Cultures with spontaneously immortalized cells were diluted to a starting density of 5 x 10⁵ cells/ml and passaged every 5–6 days in RPMI-1640 supplemented with 10% FBS and no cyclosporin.

- **Immortalization of marmoset lymphocytes by EBV.** PBMCs from seven animals with no or low detectable EBV antibody were infected in vitro with EBV (strain B95-8) (Jenson & Ench, 2002). Cells were pelleted and 3-6 x 10⁶ cells were incubated with 10⁶ TDF0 filtered EBV (strain B95-8) in 1 ml medium. Virus was allowed to adsorb to the cells for 90 min at 37 °C in 5% CO₂ after which growth medium containing 0.2 µg/ml cyclosporin was added and the cultures were maintained for evidence of cell immortalization. Seven immortalized cell lines (designated M57/B95-8, M184/B95-8, M244/B95-8, M11340/B95-8, M11344/B95-8, M11345/B95-8, M11348/B95-8) were derived, including six cell lines from common marmosets that were seronegative for EBV and Herpesvirus papio (animals 57, 184, 244, 11340, 11344, 11345) and one cell line from a common marmoset that had an antibody titre of 1:5 to EBV and Herpesvirus papio (11348).

- **Electron microscopy.** Five uninduced, spontaneously immortalized common marmoset cell lines (M11342, M11347, M12014, M12016, M12017) were washed twice in PBS and pelleted at 300 g for 5 min. To enhance herpesvirus replication, the spontaneous lymphoblastoid cell line from marmoset M117 was treated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), a known inducer of EBV (zur Hausen et al., 1978), at 20 ng/ml for 8 days and supernatant was concentrated at 40000 g for 2 h at 4 °C. Two induced, spontaneously transformed cell lines, M11341 and M11342, were pelleted after 8 days of TPA treatment. Cell and virus pellets were resuspended in 1 ml phosphate-buffered 4% formaldehyde/1% glutaraldehyde (pH 7.4), pelleted, fixed with a modified Karnovsky fixative for 2 h and rinsed in 0.1 M phosphate buffer (pH 7.4). The pellet was then treated with 1% osmium tetroxide for 30 min at room temperature and dehydrated through a series of graded ethanol washes, with a final dehydration of two changes of propylene oxide for 10 min each. Infiltration was achieved with 1:1 propylene oxide/PolyBed 812 resin for 30 min at room temperature, followed by a final infiltration of 100% PolyBed 812 under vacuum (25 psi) for 30 min. The samples were incubated at 70 °C overnight for polymerization. Blocks were sectioned using a Sorvall MT 5000 ultramicrotome for the uninduced cell lines and a Leica Ultracut microtome for the induced cell lines. Ultrathin sections (75 nm) were collected on a 150 mesh copper grid and stained with saturated aqueous uranyl acetate and Reynold’s lead citrate. Photographs were taken using a JEOL 100CX electron microscope and a Philips EM208S electron microscope (FEI).

- **Immunophenotyping.** Spontaneously immortalized marmoset cells and freshly isolated PBMCs were tested by FACS, or flow cytometry, for cell-surface markers CD2-FITC, CD3-FITC, CD4-FITC, CD8-FITC, CD11b-RPE, CD19-RPE, CD20-RPE and CD56-RPE (Becton-Dickinson).

- **PCR, DNA sequencing and Southern blotting.** In order to determine whether herpesvirus genomic sequences were present in the
cell lines and to further classify these as alpha-, beta- or gammaherpesvirus, two 5’ degenerate primers [DFASA, 5’ GTGTTCGACTCT(CT)G-C(ACTG)AG(C)TGT(CT)(ACGT)TC(CT)CC 3’, VYG, 5’ ACCTGCAG-A CGCGTGTTA(C)GAGGCAGCT(AG)CTA(AC)GTTG 3’) and one 3’ primer [GDBT1B, 5’ CGCCATGCAGAAACACG(GTGC(ACGT)ATCC(AG)GTA 3’) were synthesized, corresponding to a highly conserved region of the DNA polymerase gene of herpesviruses (Rose et al., 1997). The DFASA/GDTDB1B PCR product, since the larger product would provide more sequence information, from marmoset cell lines M117, M12017 and M11347 was amplified by PCR, separated from unincorporated nucleotides by centrifugation using a Centricon-100 column (Amicon) and cloned into a T-vector (Helena Biosciences). Plasmid DNA was prepared using Qiagen plasmid midiblocks and sequenced using a Big Dye Terminator sequencing kit on an ABI 373-S sequencer (PE Biosystems). The DNA sequences were assembled and analysed with the Wisconsin Package (version 10.0, Genetics Computer Group). This DNA sequence has been deposited with GenBank (AF291653).

Based on the results of DNA sequencing of PCR products from EBV B95-8 cells and M11347 common marmoset cells, additional PCR primers were synthesized that were specific for EBV (Baer et al., 1984) [B95-8 m1, 5’ AGGCCCTGCAAGTGGCC 3’, at positions 154549–154566 of B95-8; B95-8 m2, 5’ AGGCCTGGAGAACGCAC 3’, at positions 154934–154917 of B95-8] and the new marmoset virus (Marm1, 5’ AGTTTCTCTAAATCTGTAA 3’; Marm2, 5’ ACGCCGGGANAAAGAACGG 3’). The B95-8 primer sequences were chosen to maximize the difference between EBV and the new marmoset virus. The ‘N’ nucleotide in the Marm2 primer was because of uncertainty of this base in the initial DNA sequencing.

The corresponding Herpesvirus papio DNA polymerase sequence was obtained from the PCR products of DNA from the 594S-IX1055 cell line using the primers DFASA/GDTDB1B followed by direct sequencing using the GDBT1B primer (Rose et al., 1997).

To exclude the presence of concomitant EBV infection, either endogenous virus in the animals or possibly resulting from laboratory contamination, DNA was prepared from cell lines M11347, M11346 and M12017 and analysed for the presence of EBV BamHII W by PCR, using primers W-1 and W2-b, and Southern blotting (McClain et al., 1995).

Serological testing and cross-reactivity. Plasma from all 28 animals was tested by indirect immunofluorescence assay (IFA) for antibodies to the common marmoset virus (using the spontaneously immortalized cell line M11349 treated with TPA) and for cross-reacting antibodies to EBV (using TPA-treated B95-8, HR-1K and Raji cells), HHV-6 (using HSB-2 cells infected with HHV-6 strain DV) and Herpesvirus papio (using the 594S-IX1055 cell line).

Antibodies to common marmoset herpesvirus. Cells of the spontaneously immortalized marmoset cell line M11349 were treated with 20 ng/ml TPA, incubated for 5 days and fixed on glass slides with acetone for 5 min. Sera for all marmoset were diluted 1:5 to 1:1280 and incubated with fixed cells for 45 min followed by incubation with anti-human IgG–FITC (BioSource International) for 30 min.

Antigen cross-reaction with EBV. All spontaneously immortalized marmoset cell lines and the EBV-immortalized marmoset cell line M244/B95-8 were tested for cross-reaction with EBV latent and lytic antigens. Monoclonal antibodies (mAbs) were used to EBV p160, gp125, EA-D (p50), EA-R (p85) and gp350 (membrane antigen) in an IFA (Advanced Biotechnologies Inc.) (Jenson et al., 1999; Jenson & Ench, 2002). Normal mouse IgG (Sigma) was used as a negative antibody control. Goat F(ab’)2 anti-mouse IgG (light chain-specific) conjugated to FITC (BioSource International) was used at a 1:100 dilution.

mAbs to LMP-1 (Vector Laboratories), EBNA1 (Advanced Biotechnologies, Inc) and EBNA2 (Vector Laboratories) were used in an amplified IFA with biotin and streptavidin. Biotinylated horse anti-mouse IgG (Vector Laboratories) was used as the second antibody followed by fluorescein–streptavidin conjugate (Vector Laboratories) was used as the second antibody. Mouse IgG1 k (Sigma) was used as a negative control.

Serological cross-reaction with HHV-6. HSB-2 cells infected with HHV-6 strain DV (Salahuddin et al., 1986) were used to detect cross-reacting antibodies to HHV-6 in marmosets. Infected and uninfected cells were washed twice with PBS, spotted on glass slides, air-dried, fixed with cold acetone for 5 min and stored at —20 °C until assayed by indirect IFA (Brown et al., 1988). Marmoset serum or plasma was diluted in PBS and incubated for 45 min at 37 °C on fixed cells followed by an incubation with 1:250-diluted anti-human IgG–FITC (BioSource International) and processed as described for EBV antibodies.

Serological cross-reaction with Herpesvirus papio. Acetone-fixed smears of the 594S-IX1055 cell line (Rabin et al., 1977) were used in an indirect IFA to detect cross-reacting antibodies in marmosets to Herpesvirus papio (Jenson et al., 2000; Jenson & Ench, 2002).

EBV receptor (CD21) and CD23. Acetone-immobilized PBMCs from marmoset M117, M11341, M11346 and M12017 were tested by IFA for human CD21 (OKB7; Ortho Diagnostics) (Nemerow et al., 1985), the EBV receptor, and CD23 (DAKO) (Thorley-Lawson et al., 1995), which is strongly expressed on EBV-transformed B lymphoblasts, using the biotin–streptavidin procedure.

Immortalization of human lymphocytes by common marmoset herpesvirus. Supernatants from cell cultures of three spontaneously immortalized common marmoset cell lines (M11342, M11347, M12014) were tested in a transformation assay for immortalization of human umbilical cord lymphocytes (Lennette, 1995). Supernatants were filtered (0.22 µm), ultracentrifuged (100 000 g) for 2 h and then inoculated onto human umbilical cord lymphocytes (3·3 × 10⁶ cells in 2 ml medium). Cultures were fed twice weekly and observed for 10 weeks. Controls included supernatant from EBV B95-8 cells and human umbilical cells without virus.

DNA and protein sequence comparisons. Deduced amino acid sequences of the partial DNA polymerase gene of the new marmoset virus and other herpesviruses were aligned with Laser gene 99 (DNASTAR Inc.). In order to establish the genetic relationship of the new marmoset virus with other human and primate herpesviruses, we undertook a phylogenetic analysis by comparing the sequenced fragment of the DNA polymerase gene with sequences from other herpesviruses using the program PAUP (Sinauer Associates, Sunderland, MA). A majority-rule consensus tree was computed from 100 bootstrap replications.

Results

Cell cultures for spontaneous lymphoblastoid cell lines

Cultures of common marmoset PBMCs were evaluated weekly. Viable cell counts in the non-immortalized cell cultures decreased steadily over time. This occurred as early as 12 weeks (animals 11343 and 11348) or as late as 31 weeks (animals 11340, 11345 and 12015). The latter three cultures were held for 31 weeks because the remaining cells in these cultures were still viable although the total cell count decreased over time.
Table 1. Antibody titres in 28 common marmosets against the common marmoset herpesvirus, EBV, HHV-6 and Herpesvirus papio

Titres were determined against the spontaneously immortalized M11349 marmoset cell line (TPA-induced); EBV (TPA-induced EBV B95-8 and HR-1K cells), EA (Raji cells) and EBNA (Raji cells); HHV-6 (HSB-2 cells infected with HHV-6 strain DV); and Herpesvirus papio (TPA-induced 5945-IX1055 cells). Spontaneously immortalized marmoset cell lines were established from ten marmosets (shown in bold).

<table>
<thead>
<tr>
<th>Colony 1</th>
<th>Marmoset M11349 cells</th>
<th>EBV B95-8</th>
<th>HR-1K</th>
<th>EA (Raji)</th>
<th>EBNA (Raji)</th>
<th>HHV-6</th>
<th>Herpesvirus papio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>100</td>
<td>20</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>34</td>
<td>80</td>
<td>5</td>
<td>5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>47</td>
<td>80</td>
<td>20</td>
<td>40</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>51</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>57</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>40</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>75</td>
<td>80</td>
<td>5</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>111</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>&lt; 5</td>
<td>10</td>
</tr>
<tr>
<td>117</td>
<td>1280</td>
<td>40</td>
<td>40</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>184</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>20</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>244</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>40</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>252</td>
<td>80</td>
<td>20</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>256</td>
<td>80</td>
<td>5</td>
<td>5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Colony 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11339</td>
<td>320</td>
<td>20</td>
<td>40</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>11340</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>11341</td>
<td>80</td>
<td>5</td>
<td>10</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>11342</td>
<td>80</td>
<td>5</td>
<td>10</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>11343</td>
<td>100</td>
<td>5</td>
<td>10</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>11344</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>40</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>11345</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>11346</td>
<td>20</td>
<td>&lt; 5</td>
<td>5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>11347</td>
<td>320</td>
<td>40</td>
<td>80</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>11348</td>
<td>320</td>
<td>&lt; 5</td>
<td>5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>11349</td>
<td>320</td>
<td>40</td>
<td>40</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>11350</td>
<td>640</td>
<td>10</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>12014</td>
<td>80</td>
<td>20</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>12015</td>
<td>80</td>
<td>20</td>
<td>20</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>12016</td>
<td>80</td>
<td>5</td>
<td>10</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>12017</td>
<td>80</td>
<td>&lt; 5</td>
<td>5</td>
<td>&lt; 10</td>
<td>&lt; 2.5</td>
<td>5</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

Spontaneously immortalized common marmoset cell lines were derived from 10 animals, including nine from marmosets from the Wisconsin Regional Primate Center (11341, 11342, 11346, 11347, 11349, 11350, 12014, 12016 and 12017) and one (117) from a marmoset from Charles River Primates, Inc. (Table 1). In contrast to the non-immortalized cell lines, these spontaneously immortalized cell lines showed significant increases in viable cell counts with appearance similar to the B95-8 lymphocyte cell line (Miller et al., 1972), the prototypic EBV-infected marmoset cell line (Fig. 1A, B).

Spontaneous transformation in the immortalized cell lines was demonstrated as early as 6–8 weeks by changes in cell morphology and growth characteristics similar to those seen in spontaneous immortalization of human PBMCs infected with EBV. Clumping of rapidly dividing cells was evident in all these cell lines, as was acid production in the cell culture medium and the appearance of blast-like cells. Some adherent cells with fibroblast-like shape were also apparent in some cultures, similar to the morphology of the B95-8 cell line (Miller et al., 1972). There was a gradual increase in cell counts as the cell lines continued to proliferate, and the concentration of FBS was reduced from 20 to 10% without any adverse effect on growth. The resulting cell lines continued to proliferate.

The incubation period necessary to identify spontaneous immortalization of the marmoset cell lines extended to several weeks: three cultures (M11346, M11347 and M12017)
showed immortalization at 9 weeks, two cultures (M11349 and M11350) at 12 weeks and at approximately 20 weeks for the remainder (M117, M11341, M11342, M12014 and M12016). All of these spontaneously immortalized cell lines have been passaged through at least 10 passages, while some are at passage 30 with continued growth similar to EBV-immortalized human lymphocyte cell lines.

TPA treatment of the cultures resulted in the majority of the cells adhering to the flask with a few cells remaining in clumps.

**Electron microscopy**

Cells from five transformed, uninduced marmoset cell lines (M11342, M112347, M12014, M12016 and M12017) were examined by electron microscopy. No virus was seen in these uninduced cell lines. Electron microscopy of cells from the spontaneously transformed common marmoset cell lines M11341 and M11342 after TPA induction showed numerous cytoplasmic icosadeltahedron structures characteristic of the herpesvirus nucleocapsid (Fig. 1C). Concentrated supernatant (from M117) revealed scattered similar virus particles with a nucleocapsid surrounded by an amorphous tegument and envelope, which is characteristic of herpesviruses (data not shown).

**Immunophenotyping**

The cells infected by the common marmoset herpesvirus were primarily B cells. Spontaneously transformed common marmoset cell lines showed comparatively mild diversity of CD expression: 2.5–7.0% CD3 in five of eight cell lines; 2.4–3.0% CD11b in three cell lines; 2% of CD2, CD3 and CD8 each in one cell line; and 2–4% of CD56 in two cell lines. By comparison, marmoset cells transformed with EBV strain B95-8 showed only 2% CD11b in one of six cell lines, but CD56 was detected in all six cell lines (mean 34.4%; range 9.5–56%).

**Immortalization of marmoset lymphocytes with EBV**

All seven cultures of PBMCs from the marmosets inoculated with EBV B95-8 yielded immortalized marmoset cell lines.
(A) DNA Sequence

<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>1</th>
<th>AA CT TAA</th>
<th>T A T TTT A A T A G G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common marmoset virus</td>
<td>201</td>
<td>C T T T T T A T T G C</td>
<td></td>
</tr>
<tr>
<td>EBV</td>
<td>201</td>
<td>GGCCTGAGGA</td>
<td></td>
</tr>
<tr>
<td>Herpesvirus papio</td>
<td>201</td>
<td>GAGGAGCG</td>
<td></td>
</tr>
<tr>
<td>Common marmoset virus</td>
<td>301</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>EBV</td>
<td>301</td>
<td>GGGCGCGCG</td>
<td></td>
</tr>
<tr>
<td>Herpesvirus papio</td>
<td>301</td>
<td>T</td>
<td>G</td>
</tr>
</tbody>
</table>

(B) Protein Sequence

<table>
<thead>
<tr>
<th>Protein Sequence</th>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common marmoset virus</td>
<td>EKGLDLRPEGSEFSLSGTFVHVFHHKNSFLASLLESWLAKRIKLLGACNPR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBV</td>
<td>EHRSLAGRPEGSEFRVTGGYRHVFHHKNSFLASLLESWLAKRIKLLGACNPR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpesvirus papio</td>
<td>GCAACAGCACGGAGAGAAGAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. (A) Comparison of 364 bp of the DNA sequence of DNA polymerase (BALF5) from EBV (Human herpesvirus 4) strain B95-8 (coordinates 154559–154922; Baer et al., 1984) to the corresponding DNA sequences of the polymerase gene of the new common marmoset herpesvirus, Callitrichine herpesvirus 3, and Herpesvirus papio (Cercopithecine herpesvirus 12) (306 bp). The DNA sequences of the three common marmoset isolates that were sequenced (M117, M12017, M11347) were identical. The EBV B95-8 DNA sequence is shown as the consensus sequence, with only the differences of the new common marmoset herpesvirus (above) and Herpesvirus papio (below) indicated. (B) Comparison of the 121 aa deduced amino acid sequence translated from 363 bp of the DNA polymerase gene of the common marmoset virus, Callitrichine herpesvirus 3, with that of the equivalent sequences of DNA polymerase (BALF5) from EBV (Human herpesvirus 4) strain B95-8 (coordinates 154559–154922; Baer et al., 1984) and the overlapping 303 bp (101 amino acids) of Herpesvirus papio (Cercopithecine herpesvirus 12) that has been sequenced. Vertical lines indicate identical amino acids. Double dots indicate conservative amino acid differences.

PCR, DNA sequencing and Southern blotting

Both the DFASA/GDTD1B and VYGA/GDTD1B primer sets (Rose et al., 1997) gave positive signals in all five cells tested (M117, M12017, M11341, M11346, M11347) by PCR, with expected products of 536 and 236 bp, respectively. Stronger signals were observed using VYGA/GDTD1B. Since the larger PCR product from the primer pair DFASA/GDTD1B would provide more sequence information, these primers were used for PCR from the marmoset cell lines M117,

*(designated M57, M184, M244, M11340, M11344, M11345, M11348)*. The morphological characteristics of these PBMCs from seronegative marmosets that were transformed in vitro were similar to those of the spontaneously immortalized marmoset cells. EBV-immortalized marmoset cells showed signs of transformation (i.e. rapid growth, clumps and acid medium) 15–21 days after infection compared with the 9–20 weeks for the spontaneous marmoset cell lines. All seven of these cell lines showed signs of immortalization in less than 4 weeks of culture.
Because this region of the DNA polymerase sequence of Herpesvirus papio was not available, a PCR of Herpesvirus papio-immortalized baboon lymphocyte cell line 594S-1X1055 was performed using primers DFASA and GDTB1B and the product was T-cloned and sequenced. The common marmoset virus and Herpesvirus papio had 74% identity in this region of DNA polymerase (Fig. 2).

PCR using the EBV primers gave a strong signal from B95-8 cells and faint signals from Herpesvirus papio 594S-1X1055 and the five marmoset cell lines tested (M117, M11341, M11346, M11347, M12017). The Marm1 and Marm2 PCR primers detected an identically sized amplification from all five common marmoset cell lines tested but not from EBV B95-8 or Herpesvirus papio 594S-1X1055 (data not shown).

Supernatants from spontaneously immortalized marmoset cell lines (M11346, M11347, M12017) from three different marmosets and from 7-week-old cell cultures showing no signs of immortalization from 15 different marmosets (M11341, M12014, M12015, M11339, M11340, M11343, M11342, M12016, M12017, M11345, M11346, M11347, M11348, M11349, M11350) were assayed by PCR in duplicate for EBV using primers for the internal repeat, *Bam*HI W (data not shown). All supernatants except one of two lanes of M11347 were negative for EBV (data not shown). Six immortalized PBMC cell lines were also assayed for EBV by PCR. Three cell lines (M11346, M11347 and M12017) with common marmoset-immortalizing virus were negative for EBV, while three marmoset cell lines immortalized by EBV (M57, M184, M244) were positive for EBV (Fig. 3). Southern blotting of total intracellular DNA from spontaneously immortalized marmoset cell lines was negative using EBV *Bam*HI W as a probe (data not shown).

### Serological testing and antigen cross-reactivity

**Antibodies to common marmoset virus.** Twenty-two of the 28 marmosets had antibodies to the common marmoset virus detected by IFA using the spontaneously immortalized cell line M11349 (Fig. 4), with positive antibody titres ranging from 1:10 to 1:1280 (Table 1). All of the 10 spontaneously
immortalized marmoset cell lines were derived from animals with antibodies to this herpesvirus.

Serological cross-reaction with EBV. Of the 28 common marmosets, 19 (68%) had cross-reacting antibodies to EBV strain B95-8 and 23 (82%) had cross-reacting antibodies to EBV strain HR-1K (Table 1). None of the animals had detectable antibodies to EBV EA or EBNA.

There was no cross-reaction of non-stimulated, spontaneously immortalized marmoset PBMCs with mAbs to EBV MA (gp350), EA-D (p50), EA-R (p85), VCA (p160), LMP, EBNA-1 or EBNA-2. However, approximately 10% of the cells from each cell line did cross-react with mAb to EBV VCA (gp125) (Fig. 5). Two of the spontaneously immortalized marmoset cell lines (11349 and 12014) were treated with TPA (20 ng/ml) for 8 days. For both cell lines, the percentage of cross-reacting cells to EBV VCA (gp125) increased from approximately 10% to approximately 20%, and approximately 10% of the total cells showed a weak positive reaction with VCA (p160).

The common marmoset cell line immortalized in vitro with EBV strain B95-8, M244/B95-8, was positive for antigen expression using each of the EBV mAbs (Fig. 6), confirming that EBV can infect and transform common marmoset lymphocytes. EBNA-1 was present as weak immunofluorescence in all cells, EBNA-2 was present as strong immunofluorescence in approximately 10% of cells and weak immunofluorescence in approximately 50% of cells and LMP1, EA-D, EA-R, VCA gp125, VCA p160 and membrane antigen gp350/220 were present as strong immunofluorescence in approximately 10% of cells.

Serological cross-reaction with HHV-6. There was a high prevalence of cross-reactive antibodies with HHV-6 (Table 1). Of the 28 common marmosets, 24 (86%) were seropositive with a geometric mean titre of 1:14 in the 24 seropositive animals.

Serological cross-reaction with Herpesvirus papio. There was a high but relatively lower prevalence of detectable serological cross-reactivity with Herpesvirus papio (Table 1). Of the 28 common marmosets, 20 (71%) were seropositive, with a geometric mean titre of 1:12 in the 20 seropositive animals.

CD21 and CD23. No specific immunofluorescence was seen with either mAb against CD21 or CD23 in the four marmoset cell lines tested. The control cells Raji, MOLT-4 and HSB-2 showed the expected results, with positive staining for CD21 and CD23 in Raji, weak staining for CD21 and absence of CD23 in MOLT-4 and absence of both in HSB-2 (data not shown).

Immortalization of human lymphocytes with marmoset virus

None of the three supernatants from cell cultures of common marmoset lymphoblastoid cell lines that were tested for immortalization of human umbilical cord lymphocytes demonstrated immortalization after 8–10 weeks. The marmoset cells were not treated with TPA and, therefore, the amount of lytic virus may have been relatively low. However, approximately 10% of uninduced cells cross-reacted with lytic EBV antigens, suggesting a significant level of spontaneous lytic replication and virus production. Control immortalization of human lymphocytes with EBV strain B95-8 was positive.

DNA and protein sequence comparisons

DNA sequence alignment showed that the common marmoset virus shared 75% DNA and 83% amino acid sequence identity with EBV strain B95-8 (Fig. 2). Phylogenetic analysis based on the 364 bp DNA polymerase gene sequences of herpesviruses was performed to generate a consensus tree (Fig. 7). These herpesviruses were clearly divided into three different groups in the phylogram, with all gammaherpesviruses in one branch. The common marmoset herpesvirus formed a sub-branch with rhesus lymphocryptovirus (Cerca-
Common marmoset gammaherpesvirus

Fig. 6. Detection of EBV antigens in M244/B95-8, peripheral blood cells from an adult marmoset (M244) immortalized with EBV strain B95-8. The cells spontaneously expressed several EBV antigens: (A) VCA (gp125); (B) VCA (p160); (C) EA-D (p50); (D) EA-R (p85); (E) MA (gp350/220); (F) LMP; (G) EBNA-1, which was present as weak immunofluorescence in all cells; and (H) EBNA-2, which was present as strong immunofluorescence in approximately 10% of cells and weak immunofluorescence in approximately 50%. Original magnification: × 400.

Pithecine herpesvirus 15) (Moghaddam et al., 1997; Rivailler et al., 2002), to which it was most closely related, but it was also closely related to EBV (Human herpesvirus 4) and Herpesvirus papio (Cercopithecine herpesvirus 12). Herpesvirus papio was more closely related to EBV than either of these two lymphocryptoviruses (McGeoch et al., 2000).
Fig. 7. Phylogenetic analysis of the partial DNA polymerase gene sequences of the common marmoset gammaherpesvirus, Callitrichine herpesvirus 3, and other herpesviruses. The division of herpesviruses into subfamilies Alphaherpesvirinae (α), Betaherpesvirinae (β) and Gammaherpesvirinae (γ) is shown. GenBank accession numbers are shown in parentheses. The majority-rule consensus tree was computed from 100 bootstrap replications. This tree was computed using parsimony and therefore the scale value of 10 means 10 steps. The DNA polymerase sequences of these three isolates (M117, M12017 and M11347) were identical to each other and also identical to sequences of viruses (Callitrichine herpesvirus 3, CalHV-3) isolated from common marmosets with B cell lymphomas, which have been sequenced partially (AF091055; Ramer et al., 2000) or completely (AF319782; Cho et al., 2001). The sequence of common marmoset virus CalHV-3 (AF291653) was most closely related to that of rhesus lymphocryptovirus (Cercopithecine herpesvirus 15) (AY037858; Rivailler et al., 2002). The sequence of Herpesvirus papio (Cercopithecine herpesvirus 12) (Fig. 2) was most similar to EBV (V01555). The herpesviruses analysed included RRV17577 (Macaca mulatta rhadinovirus 17577; a member of Cercopithecine herpesvirus 17), RRV/H26-95 (rhesus monkey rhadinovirus H26-95; a member of Cercopithecine herpesvirus 17), MGVm (macaque gamma virus strain Macaca nemestrina), MneRV2 (Macaca nemestrina rhadinovirus 2), MGVM (macaque gamma virus strain Macaca fascicularis), MndRVV2 (Mandrillus rhadinovirus 2), ChRV2 (Chlorocebus rhadinovirus 2), PanRVV2 (Pan rhadinovirus 2), RFHV-mM (retropitoneal fibromatosis-associated herpesvirus – Macaca mulatta), Cercopithecine herpesvirus 19, MndRV1 (Mandrillus rhadinovirus 1), RFHV-Mn (retropitoneal fibromatosis-associated herpesvirus – Macaca nemestrina; Cercopithecine herpesvirus 18), ChRV1 (Chlorocebus rhadinovirus 1), PanRVV1 (Pan rhadinovirus 1), KSHV (Kaposi’s sarcoma-associated herpesvirus; Human herpesvirus 8), GorRV1 (gorilla rhadinovirus 1), AHV1 (Alcelaphine herpesvirus 1), OLV1 (Ovine herpesvirus 2), PLH1 (porcine lymphotropic herpesvirus 1), PLH2 (porcine lymphotropic herpesvirus 2), HVS (Herpesvirus saimiri; Samirine herpesvirus 2), HVA3 (Ateline herpesvirus 3), HHV-68 (murine herpesvirus 68; Murid herpesvirus 4), EH2 (Equid herpesvirus 2), BHV1 (bovine lymphotropic herpesvirus; Bovine herpesvirus 6), BHV4 (Bovine herpesvirus 4), HVP (Herpesvirus papio; Cercopithecine herpesvirus 12), RhLCV (Cercopithecine herpesvirus 15), MndCMV (Mandrillus cytomegalovirus), RhHV5 (rhesus cytomegalovirus; Cercopithecine herpesvirus 8), HCMV (human cytomegalovirus; Human herpesvirus 5), MCMV (mouse cytomegalovirus; Murid herpesvirus 1), HHV7 (Human herpesvirus 7), MndHVβ (Mandrillus herpesvirus beta), HHV6 (Human herpesvirus 6), HSV-1 (Herpes simplex virus 1; Human herpesvirus 1), HSV-2 (Herpes simplex virus 2; Human herpesvirus 1), PRV (pseudorabies virus; Suid herpesvirus 1), GHV2 (Gallid herpesvirus 2), EHV1 (Equid herpesvirus 1) and BHV1 (Bovine herpesvirus 1).
**Discussion**

Several lines of morphological and molecular evidence support the presence of a new lymphocryptovirus in the immortalized cell cultures established from PBMCs of common marmosets. Firstly, there was development of typical lymphoblastoid cell lines following immortalization by the new virus, a phenomenon characteristic of other gammaherpesviruses. Secondly, the partial DNA sequence of the DNA polymerase gene was distinct from other identified herpesviruses, but had high similarity to sequences from several other gammaherpesviruses. Thirdly, virions with typical herpesvirus structure were identified by electron microscopy in cells and also in culture supernatants following TPA induction. Fourthly, currently recognized gammaherpesviruses were not detected in the cultures by DNA hybridization and PCR assays. Finally, there was low but significant antigen cross-reactivity with other herpesviruses, similar to that seen between other recognized gammaherpesviruses (Jenson, 2000). The cross-reactivity in these animals was similar in prevalence and titre to previous reports of EBV cross-reacting antibodies in common marmosets (de-Thé et al., 1980; Kalter et al., 1972). This virus has been tentatively designated Callitrichine herpesvirus 3 (Ramer et al., 2000).

The high prevalence (79%) of specific antibodies to this new common marmoset herpesvirus, with a comparable prevalence at both colonies [9/12 (75%) versus 13/16 (81%)] (Table 1), indicates that most common marmosets are infected with this herpesvirus. High prevalence of infection, with spontaneous cell lines from a portion of those infected, is similar to the experience with EBV infection in humans and Herpesvirus papio infection of baboons (Jenson, 2000). All three DNA sequences from the three marmoset virus strains tested, which included animals from both colonies, were identical. Identification of the same virus from two geographically distinct animal colonies indicates that this virus is likely to be present in many common marmosets in captivity.

There was also high prevalence of cross-reactivity to Herpesvirus papio (71%), which is not unexpected because of the similarity of the common marmoset virus, EBV and Herpesvirus papio (Fig. 7). There was also a high seroprevalence (86%) to HHV-6. It is unlikely that this common marmoset gammaherpesvirus cross-reacts serologically with HHV-6. This result suggests the possibility of another virus closely related to HHV-6 that infects common marmosets, as has been suggested by serological studies in other primates (Higashi et al., 1989).

There have been several studies of EBV infection of common marmosets (Cox et al., 1996, 1998; Emini et al., 1986; Farrell et al., 1997; Mackett et al., 1996; Wedderburn et al., 1984). The present results suggest the possibility that some of the findings of previous studies may have been confounded by the presence of an unrecognized endogenous gammaherpesvirus in the common marmoset population. Alternatively, the infectious mononucleosis-like illness with EBV infection of common marmosets may indicate that the immune response to an endogenous gammaherpesvirus does not protect against subsequent gammaherpesvirus disease if the host cells are susceptible (Emini et al., 1986; Wedderburn et al., 1984). This may have relevance for understanding the basis of multiple EBV infections in humans (Apolloni & Sculley, 1994; Sixbey et al., 1989). The presence of an antigenically similar gammaherpesvirus would likely have also impaired the ability to discern EBV infection by serological testing, if the animals were infected with this new virus. This common marmoset herpesvirus requires a very long incubation period for spontaneous passage, which may partially explain why earlier studies did not identify this virus, if the animals were infected.

The long incubation period for immortalization reflects similarity to EBV type 2, which does not immortalize lymphocytes as rapidly as EBV type 1 (Rickinson et al., 1987). These results confirm that EBV can infect common marmoset lymphocytes in the absence of the common marmoset gammaherpesvirus. EBV B95-8-transformed cell lines were developed from each of the seven animals that were seronegative for common marmoset herpesvirus, EBV and Herpesvirus papio antibodies. The CD21 EBV receptor was not detected in common marmoset cells, using the mAb OKB7, suggesting specificity of this antibody to the human EBV receptor and some differences in the cell receptor despite the relative ease of EBV infection of common marmoset cells. Herpesvirus papio, like EBV, is also capable of infecting common marmoset cells, suggesting a common mechanism for gammaherpesvirus entry into common marmoset lymphocytes (Deinhardt et al., 1978). There is also evidence of decreased tumorigenicity of EBV in common marmoset lymphoblastoid cells compared with human lymphoblastoid cell lines (Katz et al., 1994).

Analysis of the sequence of eight highly conserved herpesvirus genes has led to estimates of an evolutionary timescale for the herpesvirus family (McGeoch et al., 1995). Assuming co-speciation of virus and host lineages and a constant molecular clock, the three subfamilies (alpha, beta and gamma) of herpesviruses arose approximately 180–220 million years ago, while the divergence between gamma-1 lymphocryptoviruses such as EBV and gamma-2 rhadinoviruses such as Herpesvirus saimiri (Saimiriine herpesvirus 2) was estimated to have occurred between 93 and 108 million years ago. This is consistent with our finding of a lymphocryptovirus in common marmosets from South America, the progenitor of which may well have arisen before the separation of South America and Africa over 100 million years ago. Interestingly, our phylogenetic analysis grouped the marmoset virus most closely with the rhesus lymphocryptovirus (Cercopithecine herpesvirus 15), a virus of New World monkeys, and distinguished it from EBV and the baboon lymphocryptovirus Herpesvirus papio (Cercopithecine herpesvirus 12), which were more closely related to each other than to either the rhesus or...
common marmoset lymphocryptovirus (Fig. 7). This analysis of a fragment of the polymerase gene clearly distinguishes the major sublineages of the herpesviruses and distinguishes the lymphocryptoviruses from the rhadinoviruses in the gammaherpesvirus subgroup. The fine-detail of the branching within these subgroups is less well resolved and will require further sequence data to clarify relationships and make them more statistically reliable.

It will be of interest to determine the pathology and diseases that occur in the marmoset with acute as well as persistent infection with this new virus. The ability of EBV to infect common marmoset lymphocytes and the presence of an endogenous gammaherpesvirus provide a unique model to study the pathology and interactions of gammaherpesvirus infections.

These studies were supported by grants CA57957 and RR-13986 from the National Institute for Allergy and Infectious Diseases, National Institutes of Health, and The Cancer Research Campaign. We thank Kathleen Brasky, PhD, Scott Chambers and Thomas M. Butler, DVM, MS, for veterinary support.

References


Received 28 November 2001; Accepted 8 March 2002