Use of a BJAB-Derived Cell Line for Isolation of Human Herpesvirus 8

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Establishment of latently infected cell lines from primary effusion lymphomas (PEL) presently is the most efficient system for the propagation of clinical strains of human herpesvirus 8 (HHV-8) in culture. Here we describe a new approach to culture productively replicating HHV-8 from patient samples. A BJAB-derived B-cell line, BBF, was found to retain HHV-8 longer, to support the latent and lytic replication programs, and to produce transmissible virus. Supernatants from *n*-butyrate-treated peripheral blood mononuclear cells of 24 HHV-8-seropositive renal transplant recipients were used to infect BBF cells, and replicating virus was detected in cultures from 11 patients. Moreover, BBF cells infected with saliva strains showed a highly productive profile regardless of the initial viral load, which confirms that infectious HHV-8 can be present in saliva and also suggests that saliva strains may exhibit a high tropism for B lymphocytes. In conclusion, we established an *in vitro* system that efficiently detects HHV-8 in samples with low viral loads and that produces infectious progeny. BBF cells can be used to propagate HHV-8 from different biological samples as well as to clarify important issues related to virus-cell interactions in a context distinct from endothelial and PEL-derived cell lines.

Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma-associated herpesvirus, is a novel oncogenic herpesvirus associated with the development of Kaposi's sarcoma, primary effusion lymphoma (PEL), and the B-cell variant of Castleman's disease (for a review, see reference 18). HHV-8 belongs to the γ subfamily of herpesviruses, whose main characteristics are a restricted host spectrum, *in vivo* lymphotropism, and an *in vitro* capability of infecting cell lines of lymphocytic, epithelial, and fibroblastic origin (32). *In vivo*, the tropism of HHV-8 is not strictly limited to B lymphocytes. Indeed, HHV-8 sequences were detected not only in PEL cells and circulating CD19⁺ lymphocytes but also in monocytes and endothelial and spindle cells (5, 6, 8, 14). *In vitro*, primary human endothelial cells of microvascular and macrovascular origin (17, 19, 28, 29), keratinocytes (13), fibroblasts (34), B lymphocytes (24), and a few cell lines (2, 20, 31) were found to be permissive to HHV-8 infection. However, the percentage of infected cells is generally limited in these *in vitro* systems (19, 20, 31); and the infection was found to be stable and transmissible and to involve a high percentage of cells in only a few cases (16, 25, 28, 34). This suggests that while virus entry into cells, mediated by ubiquitously expressed surface molecules (1), is minimally restricted, HHV-8 persistence and propagation are allowed only

in specialized cell lineages. Latency was demonstrated to be the default replication program in primary endothelial cells (2, 16, 25, 28, 34) and in other human and nonhuman epithelial, endothelial, and mesenchymal adherent cell lines (2), which suggests strict control of the HHV-8 lytic cycle in these cell types. Moreover, the degree of efficiency of stable episome maintenance is also variable and depends on the cell type, as several *trans*- and *cis*-acting factors seem to be implicated in the establishment of HHV-8 latency (21, 22). Indeed, cultures of spindle cells from Kaposi's sarcoma (KS) lesions retain the virus for a limited number of passages, since their *in vitro* dedifferentiation no longer provides the cellular factors involved in episome maintenance in dividing cells. Among primary cells growing in suspension, a persistent HHV-8 infection of B lymphocytes of the peripheral blood and mononuclear cells derived from cord blood was observed only in the presence of concomitant Epstein-Barr virus (EBV) infection (4, 24).

To date, the establishment of lymphoma cell lines from rare PEL cases is the most efficient system for the maintenance and propagation of HHV-8 (14, 15). Almost 10 years after the discovery of HHV-8, a manageable system for the isolation and maintenance of HHV-8 from biological samples other than PEL is not available. To search for an *in vitro* model for HHV-8 isolation, we evaluated the susceptibilities of a panel of B-cell lines and two adherent cell lines to HHV-8 infection and found that a BJAB-derived B-cell line, BBF, is able to retain HHV-8 longer than the other cell lines, to support the latent

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and lytic programs of viral replication, and to produce transmissible virus. We therefore used this cell line to isolate HHV-8 from samples with low and high viral loads.

MATERIALS AND METHODS

Cell lines. The CRO-AP/3 PEL cell line (12) was used to maximize virus production, to prepare virus inocula, and as a positive control in indirect immunofluorescence assays (IFAs). The B-cell lines assayed for their susceptibilities to HHV-8 infection included BJAB, an EBV-negative Burkitt's lymphoma cell line; ROM-T, a spontaneous EBV-positive lymphoblastoid cell line established from a human immunodeficiency virus type 1 (HIV-1)-infected patient; and BREM and BBF, two EBV and human T-cell leukemia virus type 2b (HTLV-2b)-positive HIV-1-negative BJAB-derived cell lines obtained by cocultivation of BJAB cells with irradiated peripheral blood mononuclear cells (PBMCs) from two HIV-1- and HTLV-2b-infected subjects, REM and BF, respectively (11; unpublished data). All B-cell lines were grown in complete medium, composed of RPMI 1640 (Gibco, Invitrogen Ltd., Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS; Gibco) and 2 mM L-glutamine. The human embryonal kidney epithelial HEK 293 cell line and the rhabdomyosarcoma RD4 cell line were the adherent cell lines evaluated for HHV-8 susceptibility. These cells were grown in Dulbecco's modified Eagle medium (Sigma-Aldrich, Munich, Germany) supplemented with 5% FCS and 2 mM L-glutamine.

Induction of HHV-8 lytic cycle in the PEL cell line. CRO-AP/3 cells were seeded at a concentration of 4×10^5 cells/ml in the presence of 0.3, 1.5, 3, or 6 mM *n*-butyrate (Sigma-Aldrich) or 20 ng/ml of phorbol 12-myristate 13-acetate (TPA; Sigma-Aldrich). Supernatants were processed at 48 and 72 h after treatment, as described below, and virus production was evaluated by a semiquantitative PCR.

Processing of viral supernatants. To prepare the virus inoculum, CRO-AP/3 cells were seeded at a concentration of 4×10^5 /ml and treated with 3 to 6 mM *n*-butyrate for 48 h. The supernatants were collected and centrifuged at $200 \times g$ for 5 min. The cell pellets were discarded, and the supernatants were centrifuged at $2,800 \times g$ for 30 min to eliminate cell debris, filtered through a 0.45 μ m-pore-size filter (Millipore, Billerica, Mass.), and centrifuged at high speed ($25,000 \times g$) for 3 h at 4°C to pellet the viral particles. The pellet was suspended in RPMI medium at a volume that corresponded to 1/100 of the initial volume. To test virus production from virus-inoculated and mock-treated target cell lines as well as from *n*-butyrate-induced CRO-AP/3 cells, 2 ml of supernatants was harvested at different time points and processed as described above. To measure the amount of genome equivalents (GEs), an aliquot of the 100-fold-concentrated supernatants was incubated for 1 h at 37°C with an equal volume of 2× lysis buffer (200 mM Tris-HCl, pH 7.5, 300 mM NaCl, 25 mM EDTA, 2% sodium dodecyl sulfate, 200 μ g/ml proteinase K); the DNA was phenol-chloroform extracted, ethanol precipitated in the presence 20 μ g of glycogen (Roche, Mannheim, Germany), and diluted in water. The DNA was then subjected to a semiquantitative PCR.

DNase treatment of viral supernatants and concentrated pellets. To remove cellular DNA carryover, DNase treatment was performed on the viral supernatants, whether they had been ultracentrifuged or not, derived from induced CRO-AP/3 cells or HHV-8-infected BBF cells. Each sample was treated with 4 U of RNase-free DNase I (Takara Biomedicals, Shiga, Japan) in a 100- μ l final volume containing 40 mM Tris-HCl, pH 7, 10 mM NaCl, 10 mM CaCl₂, and 6 mM MgCl₂ for 30 min at 25°C. The enzyme was then inactivated by heating at 80°C for 10 min in the presence of 5 μ l of 500 mM EDTA.

Infection assays for assessment of HHV-8 susceptibilities of target cell lines. Short-term infection assays were carried out with unconcentrated supernatants from 3 mM *n*-butyrate-induced CRO-AP/3 cells at final multiplicities of infection (MOIs) ranging from 2.5 to 5. To evaluate the cytopathic effects due to the chemical treatment, mock supernatants, prepared from 3 mM *n*-butyrate-treated BJAB cells, were used in parallel in each infection experiment. The target B-cell lines (ROM-T, BJAB, BBF, and BREM cells) were suspended at the optimal concentration (4×10^5 to 7×10^5 cells/ml) in 10 ml of virus- and mock-infected supernatants. Semiconfluent adherent cell lines, seeded 1 day before infection (3×10^4 cells/cm² of RD4 cells and 5×10^4 cells/cm² of HEK 293 cells), were exposed to 3 ml of virus- and mock-infected supernatants. After 12 h, the cells were washed twice to remove the inoculum and appropriate, fresh complete medium was added to all cell lines. Viability, the presence of morphological changes, and the persistence of viral DNA were evaluated after 24, 48, and 72 h from exposure to the viral and mock supernatants. Each experiment was set up in triplicate and was performed three times.

Long-term infection experiments were performed on two B-cell lines (BJAB

and BBF cells) and the two adherent cell lines (HEK 293 and RD4 cells). To test increasing MOIs and to remove the cytotoxic effects of *n*-butyrate derivatives, virus stocks were obtained by ultracentrifugation of the supernatants from 6 mM *n*-butyrate-treated CRO-AP/3 cells, as described above. Each cell line was exposed to concentrated supernatants, which contained virus at concentrations corresponding to final MOIs of 0.25, 2.5, and 25 GEs/cell, in the presence of complete medium for 12 h, and then washed twice and suspended in complete medium. The presence of morphological changes and viral sequences was evaluated at 2, 5, 12, and 15 days postinfection (p.i.). Each experiment was set up in duplicate and was repeated twice.

Short-term infection assays of BBF cells with high MOI (range, 20 to 120) were carried out to evaluate the expression profile of HHV-8 in this cell line. After 5 h of exposure to 3 ml of concentrated supernatants, complete medium was added to 10^7 BBF cells to reach the optimal cellular density. The cells were subsequently analyzed for the presence of HHV-8-specific mRNAs and proteins daily during the first 3 days and then every 2 days for 10 to 12 days.

Biological samples. PBMCs were isolated from EDTA-treated blood of 24 HHV-8-seropositive renal transplant recipients by Ficoll-Hypaque discontinuous gradient centrifugation (Lymphoflot; Biotest AG, Dreieich, Germany), as specified by the manufacturer. To induce the lytic cycle of HHV-8, the PBMCs were treated with 3 mM *n*-butyrate (Sigma-Aldrich) for 48 to 72 h. Saliva samples were obtained from patients affected by classic KS (one patient) and AIDS-associated KS (seven patients).

Infection of BBF cells with saliva samples. Saliva samples (100 μ l) were diluted in 3 ml of RPMI medium, centrifuged at $2,800 \times g$ for 30 min to eliminate cells, and filtered with a 0.22- μ m-pore-size filter to ensure a sterile and cell-free inoculum. Culture volumes were readjusted to 5 ml; appropriate amounts of FCS and glutamine were added to keep the cells in complete medium. BBF cells were kept in this inoculum for 3 days and then washed twice, put in culture with complete medium, and split 1:3 every 2 to 3 days for 15 days. Supernatants were collected from the BBF cell cultures at 3, 5, 7, 10, and 15 days p.i. and processed, as described above, to measure the numbers of virus particles produced in the culture supernatants. DNA was also extracted from 200 μ l of saliva samples that had previously been diluted with 200 μ l of RPMI, centrifuged, and filtered as described above and was used to determine the viral loads in the cell-free saliva fluids by real-time PCR.

PCR analyses and semiquantitative assay. To assess the presence of HHV-8 DNA in cells exposed to the virus inoculum, cellular DNA was extracted and analyzed by PCR and nested PCR with open reading frame 26 (ORF26)-specific and ORF25-specific primers, whose amplification conditions and sensitivities have been described previously (10). DNA extracted from ultracentrifuged supernatants from induced CRO-AP/3 cells or infected BBF cells was serially diluted 10-fold and amplified by using the outer set of ORF26-specific primers for a semiquantitative assessment of GEs/ml.

Reverse transcription-PCR (RT-PCR) assay. Total RNA from HHV-8-infected BBF was extracted by using RNeasy (Tel-Test Inc., Friendswood, Tex.), according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 1 to 2 μ g of total RNA in a total reaction volume of 20 μ l containing 50 mM Tris acetate, 75 mM KC₂H₃O₂, 8 mM C₄H₉O₄Mg, 5 mM dithiothreitol, 1 mM each deoxynucleoside triphosphate (Promega Corporation, Madison, Wis.), 15 U of reverse transcriptase (ThermoScript RT; Invitrogen), 40 U of recombinant RNase inhibitor (RNaseOUT; Invitrogen), and 100 ng of random hexamers (Invitrogen) or the appropriate antisense primer. The annealing premix containing RNA and the random hexamers or the antisense primer were preheated at 70°C for 10 min, cooled to 4°C (random hexamers) or to the appropriate annealing temperature for the antisense primer, and kept at 25°C for 5 min. After this annealing step, the remaining reagents were added and the mixture was incubated at 55°C for 2 h; the reverse transcriptase was finally inactivated by heating at 85°C for 5 min. The amplifications were carried out in a 50- μ l reaction mixture containing 3 to 5 μ l of cDNA, 5 μ l of 10× PCR buffer (which also contained 15 mM MgCl₂), 200 μ M each deoxynucleoside triphosphate, 125 ng of each primer, and 1.25 U of a modified recombinant *Taq* DNA polymerase (HotStarTaq; QIAGEN GmbH, Hilden, Germany); 1/20 of the first-round product was used in the nested PCR. The qualities and the amounts of RNA samples were checked by using primers specific for the β -actin transcript. To allow rigorous discrimination from DNA and to analyze the profile of HHV-8 expression, we designed three sets of nested primer pairs specific for spliced transcripts expressed in different phases of the viral cycle and specifically for the ORF73, ORF50, and ORFK8.1 transcripts. The ORF73 transcript was amplified with primer 73A (5'-GCCACGAAGCAGTCACGT-3'; nucleotides 127869 to 127852 of the BC-1 isolate; GenBank accession number U75698) and primer 73D (5'-GAATGGGAGCCACCGGTA-3'; nucleotides 127030 to 127047) for the first amplification and with primer 73B (5'-AGCTTGCTCCGGCTGACTT-

3'; nucleotides 127838 to 127820) and primer 73C (5'-AGGCAAGGTGTGGG GTCCA-3'; nucleotides 127109 to 127127) for the second amplification. The ORF50 transcript was amplified with primer 50.0 (5'-GGGGTAGTCTGTTGT GAGAA-3'; nucleotides 71554 to 71573) and primer 50.AS2 (5'-CATTCGAG AGGCACGA-3'; nucleotides 72826 to 72811) as the outer primers and with primer 50.1 (5'-CACAAAATGGCGCAAGATGACA-3'; nucleotides 71589 to 71611) and primer 50.AS1 (5'-GACCACATAGCGCACCAAGCT-3'; nucleotides 72770 to 72750) as the inner primers. Primer K8.1/1 (5'-AGGAAGAGC GTCCCGTGA-3'; nucleotides 76243 to 76260) and primer K8.1/A (5'-TGGA ACGCACAGGTAAAGTA-3'; nucleotides 76662 to 76643) were used as the outer primers for the amplification of a K8.1 transcript; primer K8.1/3 (5'-CCA CCACAGAACTGACCGA-3'; nucleotides 76297 to 76315) and primer K8.1/B (5'-GTCCAGCAATAAACCCACA-3'; nucleotides 76633 to 76614) were used as nested primers. After an initial 15-min DNA denaturation and *Taq* polymerase activation, 30 cycles were run at 94°C for 1 min; 56°C (for the ORF73-specific and the ORF50-specific outer primers), 58°C (for the ORF50-specific outer primers and the ORF73-specific and ORF50-specific inner primers), or 63°C (for the ORF50-specific nested primers) for 1 min; and 72°C for 1 min; these steps were followed by a 10-min extension step at 72°C. Each PCR sample (25 μ l) was analyzed by electrophoresis on a 1.6% agarose gel, and the bands were visualized by ethidium bromide staining.

Quantitative HHV-8 load analysis. Quantitative detection of HHV-8 was performed by real-time PCR. DNA was extracted from PBMCs, the supernatants from saliva, and infected BBF cultures. In each set of extractions, some HHV-8-negative samples (BJAB cells and supernatants) were included and were subsequently used as negative controls in all amplification procedures. HHV-8 was quantified by using the ORF26-specific primers and probe. Specifically, the inner set of previously described primers (10) was used in combination with the probe 5'-CGCTATTCTGCAGCAGCTGTTGGTGTAC-3'. The amount of genomic DNA was assessed by using *env-3* as a reference gene (35). The probes were synthesized and dual labeled by Eurogentec (Seraing, Belgium) with the fluorescent reporter dye 6-carboxyfluorescein at the 5' end and the dark quencher dye 4-(4'-dimethylaminophenylazo)benzoic acid at the 3' end to reduce the background emission from the unhybridized probe. Quantitative PCR assays and data acquisition were carried out with the ABI PRISM sequence detection system 7700 (Applied Biosystems), and reagents were supplied in the TaqMan reaction system (Eurogentec). A reaction volume of 50 μ l contained 1 μ g of each tested DNA; 5 μ l of 10 $\times$ reaction buffer; 3.5 mM MgCl₂; 200 μ M dATP, dCTP, and dGTP and 400 μ M dUTP; a 300 nM concentration of each primer; 200 nM dual-labeled probe; 1.25 U of Hot GoldStar enzyme; and 0.5 U of uracil *N*-glycosylase (UNG; Eurogentec). After an initial 5-min incubation at 50°C followed by 10 min of UNG inactivation and DNA denaturation at 95°C, 45 cycles were run at 95°C for 15 s and at 58°C for 1 min. A previously described ORF26 plasmid (10) was serially diluted in a carrier HHV-8-negative DNA (extracted from BJAB cells), and several aliquots of serial dilutions from 220,000 to 2.2 plasmid copies in 1 μ g carrier DNA were prepared once and stored at -20°C. The standard curve used to determine the amount of cellular DNA was prepared by diluting normal human genomic DNA (Novagen, Madison, Wis.) in distilled water from 1 μ g to 62.5 ng, which corresponds to 140,000 to 8,750 cells; aliquots were prepared once and stored at -20°C. All standard dilutions, controls, and samples were run in triplicate, and the average value of the copy number was used to quantify both HHV-8 genomic copies and cellular DNA. Each sample analysis was repeated three times. DNA extracted from ultracentrifuged culture supernatants and from cell-free saliva fluids was diluted in 1 μ g carrier DNA before real-time PCR analysis. Standard curves for HHV-8 were accepted when the slopes were between -3.64 and -3.32, which corresponds to PCR efficiencies ranging from 88 to 100%, and when the coefficients of correlation (r^2) were >0.98. The accuracy of the procedure was also assayed with the BC-1 cell line as a quantitative control in each plate; BC-1 cells were previously shown to contain approximately 50 HHV-8 GE/cell (15, 26), and acceptable results were considered to be 35 to 65 HHV-8 copies per cell. Procedure sensitivity was tested by using dilutions of standard plasmid DNA corresponding to 11, 2.2, and 0.22 copies per reaction. Nine of nine, six of nine, and one of nine replicates of each dilution point were found to be positive, respectively. Thus, the lower limit of detection of the assay was 2.2 copies, and its sensitivity reached 100% for at least 11 copies of plasmid DNA per reaction. The intra- and interassay reproducibilities of HHV-8 quantification were evaluated by using all dilutions of the reference curve; we found that the percentage of the coefficient of variation of threshold cycle values was $\leq$ 1.46 in nine replicates of the same run and $\leq$ 2.6 among mean values of triplicates in different runs.

Indirect immunofluorescence assay. HHV-8-infected BBF cells were fixed in 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS; Oxoid, Basingstoke, England), rendered permeable with 0.2% Triton X-100

(Sigma) for 10 min, treated with 100 mM glycine, blocked, and balanced with PBS-10% FCS for 30 min. Cells were incubated with the primary antibody diluted in PBS-2% FCS for 40 min at 37°C in a humidified environment, washed three times with PBS, and incubated with the secondary antibody under the same conditions used for the first antibody. To detect latency-associated nuclear antigen (LANA), fixed cells were incubated with a rat monoclonal antibody to ORF73 (Advanced Biotechnologies Inc. [ABI], Columbia, Md.) diluted 1:8,000 and then with Alexa 488-conjugated goat anti-rat immunoglobulin G (IgG; heavy and light chains [H+L]) diluted 1:2,000 (Molecular Probes, Eugene, Oreg.). To detect viral interleukin 6 (vIL-6) and processivity factor 8 (PF8), the cells were incubated simultaneously with a rabbit polyclonal antibody to vIL-6 (ABI) diluted 1:1,000 and with a mouse monoclonal anti-ORF59 antibody (a kind gift from B. Chandran, University of Kansas Medical Center, Kansas City, Kans.) diluted 1:300. Alexa 488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes) diluted 1:300 and Alexa 594-conjugated goat anti-mouse IgG (H+L) (Molecular Probes) diluted 1:1,000 were used as secondary antibodies. The mouse monoclonal anti-ORF59 antibody (ABI) was used at a dilution of 1:200 in combination with an Alexa 488-conjugated goat anti-mouse IgG (H+L) (Molecular Probes) diluted 1:1,000. Stained cells were viewed and counted on a fluorescence microscope (Carl Zeiss, Jena, Germany). The positivity rates were determined by counting the positively and negatively stained cells in randomly and blindly selected fields for a total of at least 1,000 cells. The cells were also visualized by confocal microscopy with an LSM510 microscope (Carl Zeiss).

RESULTS

Production of HHV-8 progeny in CRO-AP/3 cells. To set up the infection assays, we used different stimuli to maximize production of HHV-8 particles in the CRO-AP/3 PEL cell line. Treated cells were analyzed for virus production in culture supernatants after 48 and 72 h by a semiquantitative PCR. Supernatants from noninduced CRO-AP/3 cells were found to harbor 640,000 $\pm$ 488,000 GE/ml (data not shown). Treatment with 0.3 and 1.5 mM *n*-butyrate did not substantially change the rescue of viral particles. Conversely, induction with 3 and 6 mM *n*-butyrate yielded 2.8-fold and 6.5-fold increases in the basal levels of production, respectively, after 48 h (data not shown). On the third day, virus production in the supernatants decreased, due to cell toxicity by *n*-butyrate derivatives. TPA treatment led to a 2.3-fold increase in the basal amount of virus release after 48 h, and this increased to 3.2-fold after 72 h. Infection experiments were subsequently conducted with supernatants derived from CRO-AP/3 cells treated with 3 or 6 mM *n*-butyrate for 48 h, depending on the type of infection assay.

Short-term infection experiments. To evaluate the susceptibility to HHV-8 infection of B lymphoblastoid cell lines (ROM-T, BJAB, BBF, and BREM cells) and adherent cell lines (HEK 293 and RD4 cells), we conducted short-term experiments using MOIs ranging from 2.5 to 5. Mock supernatants, prepared from 3 mM *n*-butyrate-treated BJAB cells, were used in parallel in each infection experiment to evaluate the cytopathic effects due to the chemical treatment. The presence of morphological changes, viability, and the persistence of viral DNA were evaluated 24, 48, and 72 h after exposure to the viral and mock supernatants.

Cytopathic effects clearly attributable to HHV-8 were not observed in any cell line assayed (data not shown). HHV-8 sequences were detected in the DNA extracted from all cell lines exposed to HHV-8, suggesting that all were permissive at least to the early steps of HHV-8 infection. However, HHV-8 sequences were identified mainly by a single-round PCR in BJAB and BBF cells, whereas they were amplified only by nested PCR in HEK 293 and RD4 cells; and the signal was lost

TABLE 1. HHV-8 DNA detection in short-term infection experiments

Cell line	Detection by PCR of HHV-8 DNA in three independent experiments ^a					
	24 h p.i.		48 h p.i.		72 h p.i.	
	I ^b	II ^c	I	II	I	II
ROM-T	2/3	1/1	2/3	0/1	0/3	2/3
BJAB	3/3	ND ^d	3/3	ND	2/3	1/1
BBF	3/3	ND	3/3	ND	2/3	1/1
BREM	2/3	1/1	1/3	2/2	0/3	1/3
HEK 293	0/3	2/3	0/3	1/3	0/3	0/3
RD4	0/3	3/3	0/3	2/3	0/3	0/3

^a HHV-8 DNA sequences detected in cell lines at 24, 48, and 72 h p.i. by first (I)- or second (II)-round PCR.

^b The data represent the number of positive samples/total number of samples analyzed.

^c The data represent the number of nested PCR-positive samples/number of samples found to be negative by first-round PCR.

^d ND, not done.

after 72 h. ROM-T and BREM cells showed an intermediate behavior (Table 1).

Long-term infection experiments. To further analyze the susceptibilities of the target cell lines to HHV-8 infection, we performed long-term infection experiments with the two B-cell lines (BJAB and BBF cells) found to maintain the viral genome for longer periods in the short-term infection experiments. We again tested the susceptibilities of adherent cell lines using a higher MOI. In this set of experiments, virus stocks were obtained by ultracentrifugation of 6 mM *n*-butyrate-treated CRO-AP/3 supernatants. This treatment eliminated the cytotoxic effects of *n*-butyrate and allowed us to increase the MOIs (0.25, 2.5, and 25). Although giant, ballooning cells were observed among 3 to 5% of the BJAB and BBF cells exposed to MOIs of 2.5 and 25, even 1 to 2% of negative controls and cells exposed to the lowest MOI showed this morphological alteration. These changes were thus not virus-specific cytopathic alterations, since multinucleated cells were also occasionally observed in mock- and virus-exposed HEK 293 and RD4 cells. The HHV-8 sequences amplified from the virus-exposed cell lines are shown in Fig. 1. By using the highest MOI, HHV-8 sequences were amplified by PCR from both BJAB and BBF cells until the 5th day of culture and by nested

PCR onwards to the 15th day of culture. In contrast, with a lower MOI, BBF cells were found to maintain the viral sequences longer, since a specific signal was amplified by nested PCR at 5 days p.i. by using an MOI of 0.25 and at 15 days by using an MOI of 2.5. At the highest MOI, the limited capability of adherent cell lines to maintain viral sequences was confirmed, as the signal was amplifiable by nested PCR in HEK 293 cells up to only 48 h postexposure and in RD4 cells up to 5 days.

Analysis of latent and lytic replication programs of HHV-8 in BBF cells. To evaluate the expression profile of HHV-8, infected BBF cells were assayed every 24 to 48 h for 10 to 12 days for the presence of viral transcripts and proteins. Lytic replication was also induced by 3 mM *n*-butyrate treatment in BBF cells infected for 3 days, and the same parameters were analyzed after 24, 48, and 72 h. The presence of HHV-8 transcripts characteristic of the latent phase (ORF73) and the early (ORF50) and late (ORFK8.1) lytic phases were determined by RT-PCR and RT-nested PCR. The presence of viral proteins was evaluated by IFA with monoclonal antibodies that detect proteins expressed by ORF73 (LANA), ORF59 (PF8), ORFK8.1A (gpK8.1A), and ORFK2 (vIL-6). As shown in Fig. 2, an ORF50-specific transcript was detected 24 to 48 h p.i.; a K8.1-specific transcript was amplified at 48 to 72 h p.i. and after 72 h of *n*-butyrate induction (data not shown). In addition, a 337-bp product was amplified from the viral DNA. An ORF73-specific transcript was detected throughout (Fig. 2), together with a 729-bp band that was amplified from viral DNA. These transcripts were generally detected by RT-nested PCR and less frequently by RT-PCR, indicating a low percentage of infected cells. These data indicate *de novo* viral gene expression and were confirmed by IFA experiments. A punctate pattern of nuclear reactivity typical of the LANA antigen (Fig. 3B to D) was reproducibly detected throughout the culture in a subpopulation of BBF cells, with the percentages varying from 0.9 to 4.5%, mainly starting from day 3 p.i. Reactivation of the cells with *n*-butyrate did not significantly increase the percentage of LANA-expressing cells. Analysis of vIL-6, translated from an inducible viral mRNA, revealed that its expression was restricted to a limited number of cells (0.1 to 0.8%) (Fig. 3F). PF8-expressing cells were detected in a small fraction (0.3 to 1%) of BBF cells (Fig. 3G) up to the 8th to 10th day of culture. Interestingly, double-staining studies demonstrated that 30 to 80% of the BBF cells expressing PF8 coexpressed vIL-6 (Fig. 3H) and that cells expressing only vIL-6 were very rarely detected, indicating a propensity to spontaneously progress at least toward the early lytic phase. *n*-Butyrate reactivation increased the amount of PF8-expressing cells (1.5 to 2%), but PF8- and vIL-6-coexpressing cells were rarely detected (<20% of PF8-positive cells). ORFK8.1-positive cells were detected very rarely ($\leq 0.3\%$; data not shown). This lower level of expression of a truly late gene product, with respect to the level of expression of PF8, has already been reported in infected endothelial cells (16, 28) and induced PEL cell lines (36). It was suggested that only a small subgroup of cells expressing a gene characteristic of the early lytic program is able to complete this phase. Another possible explanation of this generalized, non-cell type-specific phenomenon could lie in the different half-lives of these proteins or different degrees of antibody affinity, which impair K8.1 visualization. On the whole, these data confirmed that BBF cells were fully permissive to HHV-8, which was shown

FIG. 1. Nested PCR analysis of BJAB, BBF, HEK 293, and RD4 cell lines in long-term infection experiments with increasing MOIs. ORF26-specific primers were used to detect HHV-8 sequences in the DNA extracted from virus-exposed cell lines at 2, 5, 12, and 15 days p.i. BBF cells were found to retain HHV-8 sequences longer, since a specific signal was amplified at 5 days p.i. by using an MOI of 0.25 and at 15 days p.i. by using an MOI of 2.5. The asterisks below the 233-bp band indicate that the signal was also present in the first-round PCR.

FIG. 2. Viral gene expression in HHV-8-infected BBF cells. RT-nested PCR analyses were performed on RNA extracted from HHV-8-infected BBF cells after 1, 2, 3, 5, 7, and 9 days p.i. A 205-bp ORF50-specific transcript was amplified after 1 and 2 days p.i., and a 243-bp ORFK8.1-specific transcript was amplified after 2 and 3 days p.i. An ORF73-specific band (214 bp) was detected throughout, together with a longer product amplified from viral DNA. In fact, primers were designed to amplify a spliced product and, therefore, to discriminate any amplification product deriving from viral DNA. Lanes: M, 1-kb DNA ladder marker (Invitrogen); 1, water control; 2, nested water control; 3 to 8, RNA extracted from BBF cells after 1, 2, 3, 5, 7, and 9 days p.i., respectively; 9, noninduced (for the ORF73-specific transcript) or induced (for the ORF50- and ORFK8.1-specific transcripts) CRO-AP/3 RNA; 10, CRO-AP/3 DNA.

to establish a latent as well as a fully productive program of gene expression in a small number of cells.

Analysis of infectious viral progeny release. We next evaluated whether the viral progeny generated by infected BBF cells could be further transmitted. Uninfected BBF cells were exposed to supernatants from noninduced 6-day-infected BBF cells for 48 h and then washed, and the presence of HHV-8 transcripts and proteins was tested by RT-nested PCR and IFA, respectively, after 2, 3, 5, and 7 days p.i. As shown in Fig. 4, an ORF50-specific transcript was detected after 3 days p.i., as was a 1,162-bp product amplified from viral DNA, which was detected after 2 and 3 days p.i. HHV-8-expressing cells were readily detected by IFA (data not shown), indicating that infected BBF cells produced transmissible and replication-competent virus in amounts able to reinfect other target cells without induction of the lytic phase. Moreover, liquid nitrogen storage of infected BBF cells preserved the virus; once the BBF cells were thawed, they were found to produce virus in the

FIG. 3. Expression of HHV-8 latent and lytic proteins in infected BBF cells. (A) Phase-contrast photograph of HHV-8-infected BBF cells (B, C, and D) Presence of the punctate nuclear pattern typical of the LANA protein in some BBF cells after 2 and 3 days p.i. (Alexa 488 label, confocal images). (E) Uninfected BBF cells stained with anti-LANA monoclonal antibody (Alexa 488 label). (F and G) Double-staining IFA with anti-vIL-6 polyclonal antibody (green; Alexa 488 label) and anti-PF8 monoclonal antibody (red; Alexa 594 label). (H) Merged image of vIL-6 (green) and PF8 (red) signals demonstrating that, in infected noninduced BBF cells, almost all cells that expressed vIL-6 coexpressed PF8, thus suggesting a tendency to spontaneously progress toward the early lytic phase. Magnifications, $\times 40$.

culture supernatants, indicating that they can be used as a repository of HHV-8 strains.

HHV-8 isolation from PBMCs of renal transplant recipients. To assay whether BBF cells could be employed for HHV-8 isolation, PBMCs from three HHV-8-seropositive subjects were stimulated with 3 mM *n*-butyrate for 48 to 72 h, and the supernatants were then used to infect BBF cells. BBF cells

FIG. 4. Transmission of replication-competent virus from HHV-8-infected BBF cells. Uninfected BBF cells were exposed to supernatants from noninduced 6-day-infected BBF cells and analyzed for the presence of HHV-8 transcripts by RT-nested PCR after 2 and 3 days p.i. A 205-bp ORF50-specific transcript was amplified after 3 days p.i., in addition to a 1,162-bp product amplified from viral DNA and detected after 2 and 3 days p.i., suggesting that infected BBF cells release transmissible and replication-competent viral progeny. Lanes: M, 1-kb DNA ladder marker; 1, water control; 2, nested water control; 3 and 4, RNA extracted from reinfected BBF cells after 2 and 3 days p.i., respectively; 5, induced CRO-AP/3 RNA.

were then thoroughly washed 24 h after infection. In parallel, the PBMCs were extensively washed and cultured with appropriate medium, and the culture supernatants were assayed for virus production by use of times and modalities similar to those used for the BBF cultures. Each patient's culture was kept for 12 days, and supernatants taken after 2, 6, 9, and 12 days were tested by nested PCR for the presence of viral progeny. An HHV-8-specific PCR signal was detected by nested PCR in only one of the three PBMC cultures, and the signal was lost after the 6th day of culture. Conversely, two of the three cultures of the BBF cells exposed to supernatants from stimulated PBMCs were found to be positive for HHV-8 by nested PCR up to the 12th day of culture (data not shown). These data indicate that positivity did not originate from the initial inoculum and that BBF infection with supernatants from *n*-butyrate-stimulated PBMCs prolonged the persistence of the viral isolate in vitro. A decrease in BBF cell viability was observed due to exposure to supernatants containing *n*-butyrate; the periodic addition of uninfected BBF cells was done to partially remedy this inconvenience.

We next selected 24 renal transplant recipients found to be HHV-8 seropositive at the time of renal transplant by previously described serological analyses (10) and performed the BBF cell infection as described above. After the third passage, depending on the degree of cell viability, we induced the lytic cycle with 3 mM *n*-butyrate and tested the supernatants after 2 days. We evaluated the possibility of HHV-8 isolation from the same patient at different time points (1, 3, and 6 months) after renal transplant, and a total of 42 cultures were performed. To this end, 15 patients were analyzed at at least two sequential time points. Not less than two different supernatants were tested for each culture, for a total of 106 supernatants assayed. The data are summarized in Table 2. PCR analyses showed that 2.4% of unstimulated PBMC samples (1 of 42 PBMC samples) were found to be positive by nested PCR, indicating that for 4.2% (1 of 24) of the patients HHV-8 sequences were detectable in their PBMCs before the culture (Table 2). *n*-Butyrate treatment of PBMC samples increased the percentage of detectable sequences to 9.5% (4 of 42 induced PBMC

TABLE 2. HHV-8 DNA detection in PBMCs of renal transplant recipients using BBF cells

HHV-8 DNA source and parameter	Rate of HHV-8 DNA detection ^f
Cellular DNA	
All PBMC samples.....	1/42 (2.4)
All patients.....	1/24 (4.2)
PBMCs after induction ^a	4/42 (9.5)
All patients after PBMC induction.....	4/24 (16.6)
Culture supernatant	
All patients.....	11/24 (48) ^b
Patients analyzed^c	
1 mo after T.....	7/12 (58.3) ^d
3 mo after T.....	4/15 (26.6) ^d
6 mo after T.....	6/15 (40) ^d
All cultures.....	17/42 (40.5)
All SN ^e	22/106 (20.75)
First-passage SN.....	14/32 (43.75)
Other-passage SN.....	3/48 (6.25)
Reinduction SN.....	5/26 (19.2)

^a PBMC samples were tested for the presence of HHV-8 DNA by nested PCR after 48 to 72 h of 3 mM *n*-butyrate induction.

^b The data represent the number of patients for whom HHV-8 was detected in at least one of the BBF cell cultures performed with sequential samples/total number of patients (percent).

^c Patients were analyzed 1, 3, and 6 months after renal transplant (T).

^d The data represent the number of cultures in which HHV-8 was detected in at least one supernatant/total number of cultures tested (percent).

^e At least two different supernatants (SN) were tested for each culture.

^f The data represent the number of cellular DNA or culture supernatant samples in which HHV-8 was detected/total number of samples tested (percent), unless indicated otherwise.

samples), indicating that lytic-phase induction was effective in amplifying the viral load and led to the detection of HHV-8 sequences in 16.6% of the HHV-8-seropositive patients (Table 2). However, BBF cell exposure to supernatants from induced PBMCs led to HHV-8 detection in 11 of the 24 (48%) patients. Among the cultures performed with the 12 patients' PBMCs obtained 1 month after the renal transplant, 7 were found to be positive for viral sequences. HHV-8 was detected in 4 of the 15 patients assayed 3 months after the renal transplant and in 6 of the 15 renal recipients analyzed 6 months after the renal transplant. Therefore, the isolation rate was slightly higher when PBMCs were obtained 1 month after renal transplant. Although the differences are not statistically significant, it is conceivable that the higher degree of immunosuppression maintained during the first 4 weeks after transplant may favor HHV-8 reactivation from the peripheral blood of these transplant recipients.

As to the trend of viral production in cultures, HHV-8 was more frequently detected after the first passage on BBF cells (14 of 32 [44%] supernatants tested were found to be positive) than in the subsequent passages (3 of 48 [6.25%]; Table 2). Reinduction of the infected BBF cells was found to increase the chance of HHV-8 isolation in an additional 5 of 26 previously negative samples tested. These data suggest that virus propagation from induced PBMCs was more effective after the first passage on BBF cells and that the percentage of infected and productive cells was progressively diluted during further

FIG. 5. Kinetics of HHV-8 replication of saliva strains in BBF cells. A highly productive infection was observed in BBF cells infected with 100 μ l of cell-free saliva samples from eight KS patients; virus production was measured in culture supernatants by real-time PCR and is reported as GE/ml of supernatant. The kinetics of virus replication suggest that BBF cells can spontaneously support the lytic phase of HHV-8 infectious particles present in saliva.

passages. However, the lytic-phase induction at the end of the culture may have amplified the virus to a detectable level, further indicating that BBF cells supported HHV-8 lytic replication and excluding the possibility that detection was due to residual input.

Consequently, BBF infection with supernatants derived from induced PBMCs appeared to be significantly more sensitive for virus detection ($P = 0.03$, Fisher's exact test) than molecular analyses performed with induced PBMCs. These data indicate that BBF cells allowed the isolation of HHV-8 from PBMCs obtained from HHV-8-seropositive patients, in which the viral load is usually very low and undetectable in the majority of samples by conventional, molecular techniques.

HHV-8 isolation from saliva samples. BBF cells were infected by using 100 μ l of cell-free saliva samples from eight patients, as described in the Materials and Methods, and the kinetics of virus production in culture supernatants are reported in Fig. 5. A virus replication peak was observed at 5 days p.i. in all eight cultures, with values ranging from 390 to 85,000 GE/ml. The majority of the cultures showed a further increase in virus production after 10 days or when the culture was stopped. Since the viral load measured at 3 days p.i. could have reflected the diluted initial input, the genome equivalents in the saliva samples used for infection were quantified by real-time PCR, and the viral load (expressed as GE/ml of culture supernatant) present in the culture at the beginning of infection is also reported (Fig. 5). The GE/ml measured in 100 μ l of the saliva samples ranged from 73 to 110,000; these are expressed in Fig. 5 as the total GE in the final volume (5 ml) of the BBF cell culture. Apart from one culture, in which the amount of virus showed a decrement with respect to the initial input, the values determined on the third day of culture generally showed stable or increasing trends. At that point, all cultures were washed to eliminate the initial inoculum, and the GE/ml measured at 5 days p.i. represented the degree of HHV-8 lytic activity ongoing spontaneously in BBF cells infected with

saliva strains. In these cultures, IFA experiments showed a much higher percentage of cells expressing PF8 (2 to 4%) and vIL-6 (1.2 to 2.4%) between days 5 and 7; the less productive kinetics observed in one culture (diamond in the graph in Fig. 5) was instead reflected by a higher percentage (8 to 12%) of LANA-expressing cells (data not shown). These findings indicated that BBF cells supported HHV-8 replication from the infectious particles present in the cell-free fraction of saliva. The highly productive lytic phase observed here confirmed the high infectious potential of this fluid and that BBF cells can be used to expand and propagate primary isolates harbored in saliva.

DISCUSSION

In the present report, we describe a cell line, BBF, which supports not only the latent program of HHV-8 gene expression but also an authentic lytic replication and which thus provides an *in vitro* system for HHV-8 isolation. This cell line derives from BJAB cells, a Burkitt lymphoma-derived B-cell line negative for any known herpesvirus which had already been shown to be permissive for HHV-8 infection (1, 31). To possibly reproduce the *in vivo* "physiological" setting, we exploited a different approach from previous reports by using low multiplicities of infection and avoiding the employment of polycations, such as polybrene. The susceptibilities to HHV-8 infection of four B-cell lines and two adherent cell lines were initially assayed in short-term infection experiments. As expected, all cell lines tested were found to be permissive for at least viral entry, as assessed by HHV-8 sequence detection (Table 1). Subsequently, the BBF cell line was found to retain HHV-8 sequences longer in long-term infection experiments when it was exposed to a low multiplicity of infection (Fig. 1). This persistence was not associated with a higher level of expression of integrin $\alpha 3\beta 1$ in BBF cells, which show levels of expression of the CD49c/CD29 molecules similar to those of the parental BJAB cell line by double-staining cytofluorimetric analyses (data not shown). As already shown with other cell targets (such as primary endothelial cells), HHV-8 infection in BBF cells is transient and usually involves a low number of cells, particularly when the MOI is low and the virus source is a PEL cell line. The last point appears to be critical, since in our experiments, BBF cells were not infected at good and reproducible levels (data not shown) when supernatants from induced BCP-1 cells, another PEL-derived cell line, were used (9). This confirms the attenuation or variability of the infectious properties among strains from laboratory-passaged PEL cell lines (16). Apparently in disagreement with our data and with previous findings (1, 31), Bechtel et al. (2) did not succeed in infecting BJAB cells using concentrated supernatants from BCBL-1 in the presence of polybrene or by coculture systems with infected TIME or induced BCBL-1 cells. A simple explanation might be the timing and method chosen to assess infection. In fact, cells were exclusively examined for LANA expression during the first 48 h p.i. This may be the optimal time point and the optimal antigen to be analyzed in some cell types but not necessarily in others. In our experience, the kinetics of viral gene expression in BBF cells were found to be highly dependent on the inoculum, in terms of the MOI and the viral strain. Indeed, some infected BBF cell cultures were found to

produce viral progeny with very low levels of viral transcripts, mainly detectable by RT-nested PCR (Fig. 2), and/or extremely rare cells expressing LANA, vIL-6, or PF8. On the other hand, by IFA the majority of saliva strains reached peak expression, usually starting by day 5 p.i., and infected cells were hardly detected by IFA at day 3 p.i. Therefore, B-cell lines might show expression patterns and kinetics different from those observed in other cell types. However, it is also conceivable that the BCBL-1 strain might have developed an altered tropism for B lymphocytes but a privileged one for other cell types (2, 25). Interestingly, this phenomenon could possibly imply the same mechanism described for the alternative tropism of EBV for epithelial and B cells (7).

It is noteworthy that HHV-8-infected BBF cells showed differential patterns of expression of inducible and lytic proteins compared to those of PEL cell lines. Indeed, double-staining IFA experiments showed that infected, noninduced BBF cell cultures prevalently show PF8-expressing cells, which coexpress vIL-6 at various percentages during culture (Fig. 3). In the temporal expression of these two proteins, vIL-6 is expressed during latency and increases during the delayed-early phase of the lytic cycle; PF8 is produced simultaneously or soon after, along with the other proteins involved in DNA duplication (30). The majority of cells of noninduced PEL cell lines express vIL-6, and generally, depending on the degree of spontaneous activation of the lytic cycle peculiar to each cell line, a small fraction of them coexpress PF8. Therefore, PEL cell lines provide a cellular background that preferentially maintains latency, as do many other cell systems (2, 16, 25, 28, 34). On the contrary, the majority of HHV-8 strains, derived from patient samples as well as from CRO-AP/3 cells, in BBF cells tend to choose an expression pattern more prone to spontaneously enter the lytic replication program. Indeed, infected BBF cells were found to generate viral progeny that could be further transmitted to recipient, uninfected BBF cells. Worthy of note is that, unlike other infection systems, this occurs without chemical induction of the lytic phase, indicating that BBF cells may provide a manageable tool for HHV-8 isolation.

HHV-8 lytic replication might be more spontaneously favored in BBF cells than in the parental cell line by the concomitant presence of HTLV-2b infection. The BBF cell was derived by cocultivation of irradiated PBMCs from an HIV-1- and HTLV-2b-infected patient with BJAB cells (11), a cell line that does not support HIV-1 replication and that thus allows the selective isolation of the HTLV strain. The interaction between retroviruses and herpesviruses is well documented in the literature, and their cooperation in altering the functions of target cells and in reciprocally regulating their gene expression is important in the pathogenesis of many tumors (for a review, see reference 23). We are investigating the role of HTLV-2-associated cofactors (cellular and viral) involved in promoting HHV-8 lytic replication to possibly clarify the direct and/or indirect mechanism(s) involved in the possible cooperation between HTLV-2 and HHV-8. These analyses may also identify novel pathways involved in HHV-8 reactivation from latency in B cells. Another HTLV-2-infected BJAB cell line (BREM) did not show a degree of susceptibility comparable to that of BBF cells in short-term infection experiments (Table 1). Thus, we are analyzing HTLV-2-induced cellular factors, strain-specific determinants, and the rates of HTLV-2 replica-

tion activity in both BBF and BREM cells to identify the factors possibly associated with their different responses.

We used BBF cells to isolate HHV-8 from samples with low and high viral loads obtained from HHV-8-infected patients. The protocol set up for HHV-8 isolation from PBMC samples allowed the detection of replicating virus in 11 of the 24 HHV-8-seropositive renal transplant recipients analyzed (Table 2). Although it was used with samples from a limited number of patients, the sensitivity of this biological assay was found to be significantly higher than those of molecular techniques, such as nested PCR with freshly isolated PBMCs or stimulated PBMCs. Indeed, in our experiments, the nested PCR analyses identified sequences in only 4 and 9% of the patients, respectively, detection rates similar to those observed in the Italian general population (3).

Viral reactivation and dissemination from the circulating lymphoid reservoir are important events in HHV-8-induced pathogenesis. In our system, the chemical reactivation of HHV-8 from PBMCs resulted in a detectable productive infection of BBF cells in 40.5% of the cultures (Table 2), indicating that the degree of the response to lytic phase-inducing stimuli of the virus harbored in the peripheral reservoir is variable. Indeed, isolation of HHV-8 was found to be more frequent 1 month after the renal transplant, confirming that the degree of immunodepression of the patient, kept higher during the first weeks after transplantation, plays an important role in viral reactivation. This suggests that our cell system can be used to assess not only the presence of the infection but also the replication and reactivation potentials of HHV-8 in the transplant setting. Long-term monitoring of these patients will clarify whether this isolation assay might help in assessing the risk for KS development in renal transplant recipients.

We also demonstrated that saliva can act as a vehicle for infectious particles able to establish a productive infection in the cell type that represents the primary target in the natural history of HHV-8 infection, further confirming the infectious potential of this biological fluid. Previous data showed that saliva strains can be efficiently propagated in HEK 293 cells (33). In our model, all HHV-8 strains derived from saliva samples displayed a highly productive phenotype in BBF cells (Fig. 5), independent from the initial load of the sample, which varied from 73 to 110,000 GEs in 100 μ l of cell-free saliva. On the whole, these data indicate that saliva strains may exhibit a high tropism for B lymphocytes as well as for epithelial cells. At comparable or even much higher MOIs, viral particles derived from the PEL cell line were able to infect and persist less efficiently than those derived from saliva, since replicating virus was more difficult to detect at 15 days in the majority of BBF cell infection experiments performed with CRO-AP/3-derived concentrated supernatants (data not shown). This finding indicates that the quality of viral particles in saliva is superior to that of viral particles from PEL-derived cells. It may indeed suggest that the processes involved in the formation of mature and infectious viral particles may be cell type dependent and possibly much more efficient in the oropharyngeal epithelium than in PEL tumor cells. This observation is in line with evidence that some PEL cell lines have a highly inefficient or an impaired modality of maturation of viral particles, as demonstrated by measurement of the infectious particles per GE, estimated to be 1:2,500 in BCP-1 cells with the PFU assay on

DMVEC cells (16), and as visualized in BC-1 cells by transmission electron microscopy (27).

In conclusion, our in vitro model of HHV-8 infection in part mimics the natural history of HHV-8 infection in vivo, in which B lymphocytes in the peripheral blood provide the primary targets of viral infection and only a very small, generally undetectable fraction of them persist as a systemic reservoir. With respect to the more laborious and costly establishment and maintenance of primary cultures of endothelial cells, a continuous-suspension cell line provides an alternative, more manageable context for the propagation of different isolates and for the analysis of their biological properties in a setting different from PEL and endothelial cells. Moreover, we were able to isolate strains from different patients and different biological samples. Our preliminary data also suggest that strains derived from different body compartments may have different tropisms and replication capabilities in BBF cells. Indeed, all saliva strains were found to be the most infectious source, independent of the initial viral load, and to spontaneously establish a highly productive lytic replication, confirming that saliva is highly implicated in both the sexual and the nonsexual transmission of HHV-8.

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