Activation of Kaposi’s Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Lytic Replication by Human Cytomegalovirus

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The majority of Kaposi’s sarcoma-associated herpesvirus (KSHV)-infected cells identified in vivo contain latent KSHV, with lytic replication in only a few percent of cells, as is the case for the cells of Kaposi’s sarcoma (KS) lesions. Factors that influence KSHV latent or lytic replication are not well defined. Because persons with KS are often immunosuppressed and susceptible to many infectious agents, including human cytomegalovirus (HCMV), we have investigated the potential for HCMV to influence the replication of KSHV. Important to this work was the construction of a recombinant KSHV, rKSHV.152, expressing the green fluorescent protein (GFP) and neo (conferring resistance to G418). The expression of GFP was a marker of KSHV infection in cells of both epithelial and endothelial origin. The rKSHV.152 virus was used to establish cells, including human fibroblasts (HF), containing only latent KSHV, as demonstrated by latency-associated nuclear antigen expression and Gardella gel analysis. HCMV infection of KSHV latently infected HF activated KSHV lytic replication with the production of infectious KSHV. Dual-color immunofluorescence detected both the KSHV lytic open reading frame 59 protein and the HCMV glycoprotein B in coinfected cells, and UV-inactivated HCMV did not activate the production of infectious KSHV-GFP. In addition, HCMV coinfection increased the production of KSHV from endothelial cells and activated lytic cycle gene expression in keratinocytes. These data demonstrate that HCMV can activate KSHV lytic replication and suggest that HCMV could influence KSHV pathogenesis.
infection approaches 90% in seropositive AIDS patients and can range from 30 to 100% in posttransplant patients (9). HCMV is common in persons at risk for KS, it has been identified in KS lesions (14, 23), and active HCMV infection was reported to precede the onset of KS (50). The frequency of the association of HCMV and KS led to it being considered a possible etiologic agent of KS (14, 24), and although this was found not to be true (57), it remains possible that HCMV is an augmenting cofactor. Therefore, we have investigated possible interactions between HCMV and KSHV using a recombinant KSHV containing the green fluorescent protein (GFP) and the neo gene and have demonstrated that HCMV can reactivate KSHV from latency to productive lytic replication. This work indicates that HCMV can influence KSHV lytic gene expression and viral production and suggests that HCMV could impact diseases associated with KSHV.

MATERIALS AND METHODS

Cells. Human fibroblasts (HF) were of human foreskin origin. T24, human bladder carcinoma, and DU145, human prostate carcinoma, cell lines were from the American Type Culture Collection, Inc. (ATCC). HH, T24, and DU145 were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg of streptomycin/ml, 100 U of penicillin/ml, and 2 mM l-glutamine in a humidified 5% CO2, at 37°C incubator. BCBL-1 (45) cells were obtained from the AIDS Research and Reference Reagent Program and were cultured in RPMI medium supplemented with 10% FBS, 0.1 mg of streptomycin, 100 U of penicillin, and 2 mM l-glutamine. Human umbilical vein endothelial cells (HUVEC) were the kind gift of Patricia Moeser and John Harlan and were grown in RPMI medium supplemented with 20% FBS, 50 μg of endothelial cell growth supplement (Clonetics, San Diego, Calif./ml, 100 μg of streptomycin/ml, 100 U of penicillin/ml, and 2 mM l-glutamine, plus nonessential amino acids (Gibco BRL) on plates coated with a gelatin. Keratinocytes were from Clonetics and were cultured in KGM-2 media from Clonetics.

Recombinant virus. BCBL-1 cells were used as the source for KSHV. For the construction of recombinant virus, a 4.8-kb BamHI fragment (Fig. 1) labeled with32P as a probe as described previously (41) with the modification that the gel was dried before denaturation and neutralization. The BamHI fragment was digested with HindIII (to release the insert sequence) present in the KSHV from latency to productive lytic replication. This work indicated the association of HCMV and KS led to it being considered a possible etiologic agent of KS (14, 24), and although this was found not to be true (57), it remains possible that HCMV is an augmenting cofactor. Therefore, we have investigated possible interactions between HCMV and KSHV using a recombinant KSHV containing the green fluorescent protein (GFP) and the neo gene and have demonstrated that HCMV can reactivate KSHV from latency to productive lytic replication. This work indicates that HCMV can influence KSHV lytic gene expression and viral production and suggests that HCMV could impact diseases associated with KSHV.

KSHV virion DNA analysis. Culture media were centrifuged at 500 × g for 15 min, filtered through a 0.45-μm-pore-size filter, and then centrifuged at 15,000 × g for 3 h. The supernatant was discarded, the pellet was resuspended in 100 μl of STE and centrifuged at 500 × g for 5 min, and 8 μl was used per the following reactions. For DNase treatment, 100 μl of reaction mixture with 40 mM Tris-HCl (pH 7.7). 10 mM NaCl, 6 mM MgCl2, 10 mM CaCl2, and 2 U of Q RNase (Promega, Madison, Wis.) was incubated at 37°C for 1 h. For samples treated with protease K or NP-40, a 25-μl reaction mixture with STE was incubated with protease K (0.5 mg/ml) or NP-40 (1%), both for 30 min at 37°C. For the DNase treatment of these reactions, the mixtures were diluted to 100 μl and adjusted to 40 mM Tris-HCl (pH 7.7). 10 mM NaCl, 6 mM MgCl2, and 10 mM CaCl2, and 2 U of RNase D was added before incubation at 37°C for 1 h. KSHV DNA in each sample was detected by PCR and liquid hybridization as previously described (30).

Antibody detection of viral proteins. For the immunofluorescence assay (IFA) detection of KSHV and HCMV, proteins in HF were grown on Lab-Tek Chamber slides (Nalg Nunc International), fixed with 4% paraformaldehyde for 30 min at room temperature, treated with 1% Triton X-100 for 15 min, and rinsed twice with PBS. The slides were air-dried and then washed in TBS (0.05 M Tris-HCl (pH 7.6). Fixed slides were hydrated for 5 min in 1× TBS and then blocked by immersion in 20% goat serum (diluted in TBS) for 30 min. Slides were incubated for 1 h in a humidified environment with monoclonal antibody (MAb) 7-17 (which is reactive with HCMV glycoprotein B [8]) diluted 1:50 in TBS with 0.3% bovine serum albumin and with a protein concentration-matched immunoglobulin G3 (IgG3) isotype control (Chemicon International Inc., Temecula, Calif.) diluted 1:50 in TBS. Slides were then washed in TBS and were blocked in 20% goat serum for 10 min. Biotinylated goat anti-mouse antibody [BGAM; F(ab’)2, Vector Laboratories, Burlingame, Calif.] diluted 1:200 was applied, and slides were incubated as above for 10 min and then washed with TBS. Avidin-labeled Cy5 (diluted 1:750) was applied, and the slides were incubated in the dark for 10 min. Slides were washed in TBS, and free avidin and biotin sites were blocked using two cycles of the Avidin/Biotin Blocking kit (SP-2001; Vector Laboratories). The circles were stained with 1× TBS. A second round of staining was done by first blocking with goat serum for 10 min and then incubating the slides for 30 min at 37°C with MAB 11D1 (10), diluted 1:8 in TBS with 0.3 BSA, a protein concentration-matched IgG2a isotype control and 20% goat serum for 10 min. BGAM diluted 1:250 was applied, and slides were incubated as above for 10 min and then washed with TBS. Streptavidin-labeled Alexa 594 (Molecular Probes, Eugene, Oreg.) diluted 1:100 was washed in TBS, then blocked in 20% goat serum for 10 min. Slides were then washed with TBS and counterstained with 1 μg of 4’,6’-diamidino-2-phenylindole (DAPI)/ml (Sigma) for 1 min. Slides were washed in TBS and coverslipped using the Prolong Antifade kit (Molecular Probes).

For the visualization of ORF 73 protein, cells were fixed and reacted with rabbit polyclonal antibody to ORF 73 protein as previously described (35). This was followed with biotin-conjugated F(ab’)2 fragment goat anti-rabbit IgG (Jackson ImmunoResearch Inc., West Grove, Pa.) diluted 1:200 for 30 min. at 37°C. The samples were washed twice with PBS and reacted with Alexa 594 streptavidin for 10 min at 37°C and then were washed twice with PBS and counter
stained with 1 μg of DAPI/ml for 1 min. Slides were washed in phosphate-buffered saline and coverslipped using the Prolong Antifade kit.

**RESULTS**

Recombinant KSHV. To facilitate the identification of KSHV-infected cells, a recombinant KSHV containing the GFP gene expressed by the elongation factor 1-α (EF-1α) promoter and the neo gene (conferring resistance to G418) expressed by the RSV promoter was constructed using the BCBL-1 cell line (45). The recombinant virus was generated with a construct, pQ152, containing the GFP/Neo cassette inserted between ORFs 57 and K9 at a site that sequence analysis indicates does not encode a gene (46) (Fig. 1A). BCBL-1 cells transfected with pQ152 and grown with G418 selection to generate recombinant rKSHV.152 virus 5 weeks post-electroporation (×100). Panel 1, phase contrast; panel 2, fluorescence. (C) Hybridization analysis, following gel electrophoresis, of DNA isolated from BCBL-1, and BCBL-1 with rKSHV.152, digested with BamHI, HindIII, or PstI. Left panel: analysis with the 4.8-kb BamHI fragment labeled with 32P as a probe. Right panel: analysis of BCBL-1 with rKSHV.152 with the GFP/Neo construct labeled with 32P used as a probe. B, BamHI; H, HindIII; P, PstI. Fragment sizes predicted from the BC-1 sequence (46) are as follows: BamHI, 4,774 bp; HindIII, 2,030 and 3,001 bp; and PstI, 5,907 bp. The predicted bands from a correct recombination event with the addition of the 2.7-kb GFP/Neo cassette are marked by an (*). (D) Hybridization analysis of DNA isolated from HF infected with rKSHV.152 following digestion with BamHI, HindIII, or PstI and gel electrophoresis. Left panel: autoradiogram of HF/rKSHV.152 DNA hybridized with the 32P-labeled KSHV BamHI 4.8-kb fragment. Right panel: autoradiogram of HF/rKSHV.152 DNA hybridized with the 32P-labeled GFP/Neo as a probe. B, BamHI; H, HindIII; P, PstI. Fragment sizes expected for a correct recombination event would be 7.5 kb for BamHI, 5.7 kb for HindIII, and 8.6 kb for PstI, based on the published sequence of BC-1(46). (E) 293 cells inoculated with virus isolated from BCGL-1 cells containing rKSHV.152. Infected 293 cells were examined for GFP expression and the expression of ORF 59 protein using the MAb 11D1 visualized with Alexa 594 (red). Shown are photomicrographs of infected 293 cells 2 dpi (×100). Panel 1, phase contrast; panel 2, fluorescence with filters for GFP; panel 3, fluorescence with filters for Alexa 594 (red); panel 4, merged image from the green and red filters.

Stained with 1 μg of DAPI/ml for 1 min. Slides were washed in phosphate-buffered saline and coverslipped using the Prolong Antifade kit.

**FIG. 1.** Recombinant KSHV. (A) Top, schematic diagram of the KSHV genome (46). Bottom, components of pQ152, which was used to construct recombinant virus. An expanded segment of the KSHV genome shows the 4.8-kb BamHI fragment containing ORFs 57 and K9. This fragment was used for the insertion of the GFP/Neo cassette between the polyadenylation sites for ORFs 57 and K9. (B) Photomicrographs of BCBL-1 cells that were transfected with pQ152 and grown with G418 selection to generate recombinant rKSHV.152 virus 5 weeks post-electroporation (×100). Panel 1, phase contrast; panel 2, fluorescence. (C) Hybridization analysis, following gel electrophoresis, of DNA isolated from BCBL-1, and BCBL-1 with rKSHV.152, digested with BamHI, HindIII, or PstI. Left panel: analysis with the 4.8-kb BamHI fragment labeled with 32P as a probe. Right panel: analysis of BCBL-1 with rKSHV.152 with the GFP/Neo construct labeled with 32P used as a probe. B, BamHI; H, HindIII; P, PstI. Fragment sizes predicted from the BC-1 sequence (46) are as follows: BamHI, 4,774 bp; HindIII, 2,030 and 3,001 bp; and PstI, 5,907 bp. The predicted bands from a correct recombination event with the addition of the 2.7-kb GFP/Neo cassette are marked by an (*). (D) Hybridization analysis of DNA isolated from HF infected with rKSHV.152 following digestion with BamHI, HindIII, or PstI and gel electrophoresis. Left panel: autoradiogram of HF/rKSHV.152 DNA hybridized with the 32P-labeled KSHV BamHI 4.8-kb fragment. Right panel: autoradiogram of HF/rKSHV.152 DNA hybridized with the 32P-labeled GFP/Neo as a probe. B, BamHI; H, HindIII; P, PstI. Fragment sizes expected for a correct recombination event would be 7.5 kb for BamHI, 5.7 kb for HindIII, and 8.6 kb for PstI, based on the published sequence of BC-1(46). (E) 293 cells inoculated with virus isolated from BCGL-1 cells containing rKSHV.152. Infected 293 cells were examined for GFP expression and the expression of ORF 59 protein using the MAb 11D1 visualized with Alexa 594 (red). Shown are photomicrographs of infected 293 cells 2 dpi (×100). Panel 1, phase contrast; panel 2, fluorescence with filters for GFP; panel 3, fluorescence with filters for Alexa 594 (red); panel 4, merged image from the green and red filters.
BCBL cells, and the fragment sizes predicted by a correct recombination event were the predominant bands (Fig. 1D).

To test for infectious recombinant virus, cells containing rKSHV.152 were induced with TPA, and virus isolated from the culture medium was used to infect 293 cells. Inoculated cells were observed by fluorescence microscopy, and 1 dpi, 293 cells expressing GFP were evident. To confirm that the GFP expression was indicative of KSHV infection, cultures were assessed for expression of both the GFP and the KSHV ORF 59 lytic nuclear protein using MAb 11D1 (10) 2 dpi (Fig. 1E), and it was found that ORF 59 expression and GFP could be localized to the same cells (Fig. 1E, panel 4). There were also cells that expressed ORF 59 that were not GFP positive, as would be expected due to the fact that wild-type virus was present. This demonstrated that the BCBL-1 cells containing rKSHV.152 could produce infectious recombinant virus that expressed GFP upon infection of susceptible cells.

Establishment of KSHV latently infected cultures. In cells that are permissive for the expression of the EF-1α/GFP gene, rKSHV.152 provided a means of determining the susceptibility of cells to KSHV infection by the expression of GFP. In addition, the neo gene allowed the selection of infected cells with G418. Figure 2A shows three cell types, T24, a human bladder carcinoma, DU145, a human prostate carcinoma, and human fibroblasts (HF), which expressed GFP following inoculation and could subsequently be cultured with G418 selection. These cells showed no cytopathic effect (CPE) upon infection with KSHV, and no ORF 59 expression or infectious virus could be detected (data not shown). Long-term cultures (defined as at least six passages with cells split one to three with G418 selection) of rKSHV.152-infected HF, T24, and DU145 cells could be maintained which were KSHV DNA positive by PCR, but no CPE was evident and no infectious virus could be detected in culture supernatant or sonicated cells (data not shown). Because these features were indicative of a latent infection, it was of interest to analyze these cells for KSHV latent gene expression and for the structure of the viral DNA present.

A latency-associated nuclear antigen (LANA) has been detected in BCBL cells using sera from KS patients (21), and it was later identified to be encoded by ORF 73 (42). To determine if ORF 73 protein was expressed in rKSHV.152-infected HF cultures, cells were examined by IFA for expression of ORF 73 using rabbit polyclonal antiserum raised against ORF 73, and the punctate nuclear pattern typical of ORF 73 was detected (Fig. 2B). Essentially all HF cells were positive for ORF 73, and the same result was found for the T24- and DU145-rKSHV-infected cells (data not shown).

The structure of the KSHV genome present in long-term cultures was analyzed by Gardella gel analysis. Gardella gels separate circular viral genomes, present during latent replication, from linear viral DNA present during lytic replication (22), as used to analyze KSHV in BCBL-1 cells (44). Gardella gel analysis of T24, DU145, and HF cultures containing rKSHV.152 demonstrated that these cells contained circular viral genomes, indicative of latent viral replication, with no detection of linear, lytic DNA (Fig. 2C). Both these results were consistent with KSHV latent infection.

Activation of KSHV lytic gene expression by HCMV. The establishment of cells containing only latent KSHV, particularly primary HF cultures, offered an experimental system for studying the reactivation of KSHV. HF are susceptible to infection by a number of viruses, including HCMV. HF with rKSHV.152 were susceptible to HCMV infection, demonstrating typical CPE (Fig. 3A). In addition, following HCMV infection the majority of cells demonstrated GFP expression, whereas in latent cells only 25 to 30% of cells were GFP positive. This indicates that in most latent cells the EF-1α promoter is inactive, perhaps due to methylation, which has been shown to influence gene expression in Epstein-Barr virus (2) but is activated by HCMV. To determine if HCMV infection led to the expression of KSHV lytic cycle proteins, HF/rKSHV.152 cultures, without or with HCMV infection, were reacted with MAb 11D1 for the detection of the KSHV lytic ORF 59 protein. While no ORF 59 protein was seen in cultures without HCMV, approximately 20 to 25% of cells 2
FIG. 3. Detection of viral proteins. (A) HF infected with rKSHV.152 and HF coinfected with rKSHV.152 and HCMV, photographed with phase contrast or fluorescence. Shown for HF/rKSHV.152 are phase contrast (panel 1) and fluorescence (panel 2) (×100). Shown for HF/rKSHV.152 infected with HCMV are phase contrast (panel 3) and fluorescence (panel 4). (B) Expression of the KSHV lytic ORF 59 protein induced by HCMV. HF infected with rKSHV.152, minus and plus HCMV infection, were analyzed for the presence of the lytic cycle 59 protein with MAb 11D1 visualized with Alexa 594 (red) (panel 2), and stained with DAPI (blue) (×200). Panel 1, HF with rKSHV.152; panel 2, HCMV-infected HF, and panel 3, infected with HCMV. (C) Identification of ORF 59 expression in HF coinfected with rKSHV.152 and HCMV. HF latently infected with rKSHV.152 were infected with HSV-1 and 2 days later were examined for ORF59 expression with MAb 11D1 visualized with Alexa 594 (red) and stained with DAPI (blue) (×200). Panel 1, HF with rKSHV.152; panel 2, HF with rKSHV.152 infected with HCMV. (D) Visualization of GFP and nuclear ORF 59 protein with MAb 11D1 and Alexa 594 (red) (panel 1) or rKSHV.152 and HCMV (panel 2). (E) Identification of the KSHV ORF 59 protein and HCMV gB in fibroblasts infected by rKSHV.152 and HCMV. Photomicrographs show the detection of GFP expression (panel 1), KSHV ORF 59 protein with MAb 11D1 visualized with Alexa 594 (red) (panel 2), and HCMV gB with MAb 7-17 detected with Cy5 (blue) (panel 3). Panel 4, merged image showing ORF 59, gB, and GFP in coinfected cells (×600); panel 5, HF infected with HCMV reacted with antibodies 7-17 (blue) and 11D1 (red), demonstrating that 11D1 did not react with an HCMV-infected cell; panel 6, BCBL-1 cells induced with TPA for 2 days and reacted with MAb 11D1 (red) and MAb 7-17 (blue), showing that 7-17 does not react with KSHV proteins; panel 7, HF infected with rKSHV.152 reacted with MAb 11D1 and MAb 7-17.

Production of infectious KSHV induced by HCMV infection.

The expression of GFP by rKSHV.152 upon the infection of cells facilitated the detection of infectious virus. To determine if infectious KSHV was produced by HF cultures coinfected with KSHV and HCMV, virus was harvested from coinfected cultures 3 days post-HCMV infection and was used to inoculate 293 cells. This resulted in GFP-positive 293 cells (Fig. 5A), demonstrating the presence of infectious rKSHV.152. ORF 59-positive cells detected using MAb 11D1 were also found in these infected 293 cultures (data not shown). Next, the temporal production of KSHV by coinfected cells was determined.
by the infection of 293 cells with virus harvested at time points post-HCMV infection, followed by the determination of the number of GFP-positive 293 cells. No KSHV was detected without HCMV infection at 1 day post-HCMV infection or in cells infected with UV-inactivated HCMV, but infectious rKSHV.152 was detected 2 days post-HCMV infection (Fig. 5B). Infectious HCMV was first present at 3 dpi, as is the case for HF without KSHV (data not shown). The effect of the HCMV MOI on KSHV production was also examined, and it was found that the activation of KSHV by HCMV is achieved at a low MOI, indicating that a few or 1 PFU per cell can reactivate KSHV (Fig. 5C). A high HCMV MOI reduced the level of KSHV, possibly due to the significant CPE. In contrast to HCMV, HSV which activated ORF 59 expression did not result in the production of infectious KSHV (data not shown). Whether this is due to the more rapid replication of HSV interfering with KSHV, or the failure of HSV to activate the entire KSHV replication cycle or supply a necessary function, is not known.

**Activation of KSHV by HCMV in endothelial cells.** The finding that HCMV could activate KSHV in cells of epithelial origin made it of interest to determine if this occurred in other cell types infected by both viruses. Endothelial cells are a significant cell type present in KS lesions that are KSHV positive (7). Because long-term HUVEC cultures demonstrating only latent replication have not been established, these experiments were carried out in cultures infected first with rKSHV.152, followed 2 h later by HCMV infection with strain VHL/E...
showed a low percentage of cells with nuclear expression of ORF 59 as detected by MAb 11D1, signifying lytic protein expression (Fig. 6A, panel 1). With HCMV coinfection, there was a significant increase in ORF 59-positive nuclei (Fig. 6A, panel 2), demonstrating that HCMV could activate lytic cycle gene expression in HUVEC. HCMV coinfection increased KSHV lytic DNA replication, as demonstrated by Gardella gel analysis comparing HUVEC infected with rKSHV.152 to HUVEC coinfected with HCMV and rKSHV.152 (Fig. 6B), where a significant increase in linear KSHV DNA was present with HCMV infection. To investigate if HCMV affected the production of KSHV by endothelial cells, the amount of rKSHV.152 produced by HUVEC, without and with HCMV infection, was determined by inoculating 293 cells with virus harvested from the HUVEC cultures 3 dpi (Fig. 6C). An approximately threefold increase in the level of infectious KSHV was noted, but this was well below the increase in the number of ORF 59-positive nuclei induced by HCMV, suggesting that many cells that express KSHV early lytic proteins may not go on to produce virus. It should also be noted that we examined HUVEC from seven different donors; and while infection with KSHV, lytic gene expression, and induction of lytic gene expression by HCMV were similar, the production of KSHV varied significantly. Two lines were relatively good producers (one is presented in Fig. 6), three produced barely detectable virus, and two were intermediate (J. Vieira, unpublished observations). The reasons are at present unknown.

**DISCUSSION**

In this report we have shown that HCMV, a common pathogen in immunosuppressed individuals, can activate KSHV lytic replication. Important to this investigation was the construction of a recombinant KSHV carrying the GFP and neo genes, rKSHV.152. In cells permissive for expression of the EF-1α promoter, GFP expression was a sensitive and specific marker for cells that could be infected by KSHV, which made it particularly useful for KSHV, where infection is often without CPE. This included 293, HF, HUVEC, T24, and DU145 cells. The neo gene present on rKSHV.152 made it possible to select for infected cells with G418 and allowed the first establishment of cultures, including primary HF and DU145 cells, where all cells contained only latent KSHV. DU145 cells had previously been reported not to support latent replication in a PCR-based study (36). Cell lines containing only latent KSHV will be of value in studies of viral and cellular gene expression during latency and of factors that contribute to virus reactivation.

The establishment of HF with latent KSHV provided a system for examining the interaction between KSHV and HCMV. This resulted in the demonstration that the infection of KSHV latently infected HF with HCMV, reactivated KSHV lytic replication, and resulted in the production of infectious KSHV. For this study, GFP expression by rKSHV.152 facilitated the detection of infectious virus and enabled a quantitation and temporal analysis of virus production. Studies on the activation of KSHV lytic replication have identified the ORF 50 gene as an immediate-early gene, with homology to the EBV Rta gene, that can activate lytic viral genes when expressed from transfected recombinant constructs (55). The process that HCMV may invoke in the reactivation of KSHV is not known and is currently under investigation. It is of note that HCMV is a betaherpesvirus and in this case reactivates KSHV, a gammaherpesvirus. Cross-reactivation between herpesviruses has been examined in a limited number of studies. In cells coinfected with KSHV and EBV, cross-reactivation between these two gammaherpesviruses was not found (55). Reactivation of HHV6 by HHV7 has been reported for these similar betaherpesviruses (27). The activation of a gammaherpesvirus by a betaherpesvirus has been reported for the reactivation of EBV by HHV6 (18), and as with KSHV and HCMV, coinfected cells were identified and infectious virus was necessary.

KS lesions are in large part derived from endothelial cells,
and endothelial cells are an important site of HCMV replication, which makes it of interest to examine the potential for KSHV-HCMV interaction in endothelial cells. In HUVEC a low level of lytic KSHV replication was shown by ORF 59 expression and the production of infectious rKSHV.152. With HCMV infection, an approximately 10-fold increase in the expression of the KSHV lytic ORF 59 protein was observed; however, the increase in infectious rKSHV.152 was only approximately three fold, suggesting that many cells with early lytic gene expression may not go on to a productive infection. A greater percentage of cells expressing early genes than late genes was found in microvascular endothelial cells (35). In vitro KSHV infection of both macrovascular and microvascular endothelial cells has been reported, with viral production in microvascular cells (19, 35, 40, 43). This report demonstrated HCMV activation of KSHV in HUVEC, which are macrovascular cells, and similar results have been seen with dermal microvascular endothelial cells (J. Vieira, unpublished observations). Besides any effect of increased virus production, the increase in lytic gene expression induced by HCMV could in itself have an impact in KS. Some of the viral genes indicated for roles in angiogenesis, cell proliferation, signal transduction, immune modulation, and inflammatory infiltration (49) are expressed as lytic genes (47), suggesting that lytic gene expression induced by HCMV could augment these processes.

While KSHV and HCMV coinfectected cells have not been identified in vivo, there are multiple cell types with potential for KSHV and HCMV interaction. The cellular tropism for KSHV is proving to be broad; the cell types infected by KSHV include endothelial cells, fibroblasts, keratinocytes, B cells, monocytes/macrophages, and glandular epithelial cells (5, 7, 13, 15, 26, 32). This is a cellular tropism shared in large part by HCMV, illustrating a wide variety of sites for possible interaction. Monocytes are considered sites of latency for both viruses, and in vitro studies have indicated that cytokines induced by immune activation can lead to reactivation of KSHV and HCMV (33, 52). HCMV is found in KS lesions, and the endothelial/spindle cell of KS lesions could be a target cell for both viruses. HCMV and KSHV are both shed in saliva (6, 9, 30, 59). Although HCMV replication is predominately found in salivary glands, and the data suggest that KSHV is not in the salivary gland but replicates in oral epithelial (12, 59), in immunosuppressed patients HCMV replication can be found in the oral mucosa (48).

The possible clinical implications for the interaction between HCMV and KSHV are unknown and in need of further study, but a number of considerations exist. The diseases associated with KSHV and HCMV occur in the same patient populations, and active HCMV replication can be common in those associated with KSHV and HCMV occur in the same patient populations, and active HCMV replication can be common in KS patients. In a study of 23 men with KS or a history of KS, 11 had HCMV viremia, 7 had KSHV viremia, and 5 were positive for both as determined by PCR detection of viral DNA in serum (J. Vieira, unpublished data). The activation of KSHV lytic replication by HCMV could have multiple effects on KSHV-related diseases. The induction of KSHV lytic proteins, which are thought to play a part in KS through promoting proliferation, angiogenesis, and inflammatory infiltration, could exacerbate lesions. The HCMV activation of KSHV from latently infected cells could generate virus capable of seeding surrounding cells with KSHV, thereby increasing the population of KSHV latently infected cells that could contribute to tumor formation. Coinfection of cells could also act to increase the viral load of KSHV.

It has become evident that KS is a multifactorial disease. The ubiquitous finding of KSHV in KS lesions demonstrates its core role in KS, but infection with KSHV by itself rarely appears adequate for KS development. Our data demonstrate that HCMV can activate KSHV lytic replication and suggest the importance of considering the potential for infectious agents present in immunocompromised patients to interact and exacerbate disease. This work has also demonstrated the utility of the rKSHV.152 virus for the determination of cells susceptible to KSHV infection, the detection of infectious KSHV, and the establishment of KSHV latently infected cells.

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REFERENCES