Activation of the Epstein-Barr Virus Replicative Cycle by Human Herpesvirus 6

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One common attribute of herpesviruses is the ability to establish latent, life-long infections. The role of virus-virus interaction in viral reactivation between or among herpesviruses has not been studied. Preliminary experiments in our laboratory had indicated that infection of Epstein-Barr virus (EBV) genome-positive human lymphoid cell lines with human herpesvirus 6 (HHV-6) results in EBV reactivation in these cells. To further our knowledge of this complex phenomenon, we investigated the effect of HHV-6 infection on expression of the viral lytic cycle proteins of EBV. Our results indicate that HHV-6 upregulates, by up to 10-fold, expression of the immediate-early Zebra antigen and the diffuse and restricted (85 kDa) early antigens (EA-D and EA-R, respectively) in both EBV producer and nonproducer cell lines (i.e., P3HR1, Akata, and Raji). Maximal EA-D induction was observed at 72 h post-HHV-6 infection. Furthermore, expression of late EBV gene products, namely, the viral capsid antigen (125 kDa) and viral membrane glycoprotein gp350, was also increased in EBV producer cells (P3HR1 and Akata) following infection by HHV-6. By using dual-color membrane immunofluorescence, it was found that most of the cells expressing viral membrane glycoprotein gp350 were also positive for HHV-6 antigens, suggesting a direct effect of HHV-6 replication on induction of the EBV replicative cycle. No expression of late EBV antigens was observed in Raji cells following infection by HHV-6, implying a lack of functional complementation between the deleted form of EBV found in Raji cells and the superinfecting HHV-6. The susceptibility of the cell lines to infection by HHV-6 correlated with increased expression of various EBV proteins in that B95-8 cells, which are not susceptible to HHV-6 infection, did not show an increase in expression of EBV antigens following treatment with HHV-6. Moreover, UV light-irradiated or heat-inactivated HHV-6 had no upregulating effect on the Zebra antigen or EA-D in Raji cells, indicating that infectious virus is required for the observed effects of HHV-6 on these EBV products. These results show that HHV-6, another lymphotropic human herpesvirus, can activate EBV replication and may thus contribute to the pathogenesis of EBV-associated diseases.

Herpesviruses are ubiquitous pathogens found throughout the animal kingdom. Infection by human herpesviruses usually occurs during childhood and generally runs a benign course. Occasionally, more serious complications are observed. Some of these herpesviruses are known by their marked lymphotropicism, as well as their association with immunopathological disorders. For example, primary infection of young adults by the Epstein-Barr virus (EBV) can lead to a self-limiting lymphoproliferative disease known as infectious mononucleosis (24, 25). Furthermore, the presence of EBV genomes has been documented in cells of anaplastic nasopharyngal carcinoma (55, 73), African Burkitt’s lymphoma (27, 42), and non-Hodgkin’s lymphomas in immunosuppressed individuals (20, 21). Transmission of EBV occurs almost exclusively through saliva. Infectious virus can be recovered from the oropharynx in the majority of infectious mononucleosis patients and from healthy seropositive individuals (17). EBV can infect, in vivo, EA-R, the lymphoid and epithelial tissues of the nasopharynx. In vitro, EBV immortalizes human and primat B lymphocytes. These lymphoblastoid cell lines (LCL) produce little, if any, infectious virus. The viral genome is maintained as an episome, and its expression is limited to a small number of nuclear and latent membrane proteins (12).

Another lymphotropic human herpesvirus gaining increasing medical attention is human herpesvirus 6 (HHV-6). It was first isolated in 1986 from peripheral blood leukocyte cultures of patients with lymphoproliferative disorders and AIDS (58). HHV-6 has been identified as the causative agent of exanthem subitum (62, 67). HHV-6 has also been associated with herpetic-negative infectious mononucleosis (60), meningococcal meningitis (31), hepatitis (4, 14), fatal hemophagocytic syndrome (29), and interstitial pneumonitis (8). Like that of EBV, transmission of HHV-6 is thought to occur through saliva, and primary infections occur within the first 3 years of life (6, 22, 50). HHV-6 can infect many cell types, most of which are constituents of the immune system (2, 40, 47). Interestingly, human B cells appear to be particularly susceptible to HHV-6 infection in vitro only if they have been previously infected with EBV (1).

A common property of herpesviruses is establishment of latency following primary infection. Consequently, these viruses have the potential to reactivate and cause serious secondary infections, especially in immunocompromised hosts. The factors which cause these viruses to reactivation are not fully known. It has been documented that in immunocompromised hosts, EBV and HHV-6 are reactivated and can be recovered from these patients at a higher frequency (3, 21, 35, 68, 69). EBV reactivation in such hosts leads to increased susceptibility to development of EBV genome-positive, non-Hodgkin-type (B-cell) lymphomas (20, 21). Indeed, experiments using the severe combined immunodeficient mouse model and inoculation with peripheral blood leukocytes from EBV-seropositive donors have also shown a correlation between EBV reactivation and the development of such lymphomas (49). Further-
more, there is evidence that EBV reactivation can be associated with severe chronic diseases (59).

Factors that influence viral reactivation include viruses themselves. The model which has been best studied is that of reactivation of human immunodeficiency virus type 1 (HIV-1) by DNA viruses (16, 28, 45, 48, 51). These DNA viruses, which include herpesviruses, activate HIV replication through trans-activation of the long terminal repeat of HIV. Furthermore, some herpesviruses can also induce the synthesis of cytokines, such as tumor necrosis factor alpha and interleukin-6, which are known to reactivate HIV (15, 19). Consequently, in HIV-infected individuals, reactivation of latent herpesviruses can lead to increased expression of HIV, which in turn may accentuate the downfall of an already impaired immune system.

Viral cofactors in the evolution of EBV-related lymphomas have not been elucidated. The detection of HHV-6 genomic sequences in some B-cell neoplasias (1, 32, 63) and the potential of an HHV-6 genomic segment in transforming rodent cells (57) suggest a possible, if indirect, role for this virus in certain cancers. Further interactions among viruses may be necessary and/or complementary in the generation of certain pathologic changes. To further our understanding and eventually prevent the occurrence of EBV reactivation, it is essential to identify the causes. This study was therefore undertaken to evaluate the influence of HHV-6, another lymphotropic human herpesvirus, on the expression of EBV replicative-cycle proteins.

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MATERIALS AND METHODS

Cell lines and culture conditions. The P3HR1, Akata, B95-8, Raji, and HSB-2 cell lines were obtained from the American Type Culture Collection (Rockville, Md.). All of these cell lines are of human origin, except B95-8, which is of marmoset monkey origin. LCL were obtained following in vitro immortalization of primary B lymphocytes with EBV (B95-8 strain). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, 30 μg of gentamicin per ml, and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer.

Monoclonal antibodies (MAbs). The following MAbs were used: BZ.1 anti-Zebra (gift from L. S. Young and M. Rowe), R3 against the diffuse early antigen (EA-D), 85K against the restricted early antigen (EA-R), 2L10 anti-gp350, L2 against the viral capsid antigen (VCA) (gift from G. Pearson), 72A1 anti-gp350 (gift from G. Hoffman), p11E8 anti-gp56/20 of HHV-6, 2D6 anti-HHV-6 gp105/82 (gift from N. Balachandran), and HAR 1-5 anti-gp110/60 of HHV-6 (gift from J. Luka). All MAbs directed against EBV proteins were tested for cross-reactivity with HHV-6 and found to be unreactive. In some experiments, the MAbs were directly labelled with fluorescein or rhodamine as follows: MAbs 2L10 and p11E8 were suspended in 0.1 M carbonate buffer (pH 9.0) at 2 mg/ml. Fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) (1 mg/ml in dimethyl sulfoxide) was added to the solution (final concentration, 50 μg/ml) and incubated overnight at 4°C. Ammonium chloride was added to a final concentration of 50 mM, and the mixture was incubated at 4°C. Xylene cyanol and erythrosin, at final concentrations of 0.1 and 5%, respectively, were added before separation of labelled MAbs by chromatography with G-50 columns.

Virus production. HHV-6 (GS strain) was propagated in HSB-2 cells as described previously (15). Briefly, HHV-6-infected HSB-2 cells were mixed with uninfected cells at a ratio of 1:10. When 75% of the cells showed cytopathic effects (CPE), as determined by light microscopy, cell-free culture fluid was harvested and filtered through a 0.45-μm-pore-size filter and the virus was pelleted by centrifugation (25,000 × g) for 90 min at 4°C. The virus pellet was suspended in RPMI 1640 and frozen at −85°C until used. The HHV-6 titers, expressed as the 50% tissue culture infective dose, was determined by scoring the number of HSB-2 cells exhibiting CPE. The virus stock used had a titer of 10^5 50% tissue culture infective doses/ml. The mock-infected control was prepared from uninfected HSB-2 culture supernatant as described above.

Infection of cell lines. Cells (2 × 10⁶ to 3 × 10⁶) were pelleted and either infected with HHV-6 (10^6 50% tissue culture infective doses/ml for 10^6 cells), mock infected, or treated with UV-irradiated HHV-6 (1 h) or heat-inactivated HHV-6 (56°C, 1 h) for 2 h at 37°C and subsequently suspended in 10 ml of culture medium. The experimental conditions for virus inactivation were the same as in our earlier studies and were found to inactivate over 99% of viral infectivity without, however, affecting the ability of viral particles to modulate cytokine gene expression when infectivity was not required (15, 19). Cells were harvested at different times, washed with phosphate-buffered saline (PBS; pH 7.4), and processed for fluorescence microscopy, Western blotting (immunoblotting), or Southern blotting.

Immunofluorescence assays. PBS-washed cells were processed for immunofluorescence by acetone fixation at −20°C. Following 1 h of incubation with the primary antibody, slides were washed and the fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (Ortho Diagnostics, Raritan, N.J.) was added for 1 h. Slides were then washed with PBS, mounted, and examined with an Axioskop fluorescence microscope (Zeiss). The percentage of positive cells was calculated after at least 200 cells had been counted. Statistical significance was determined by using a χ² test. A P value of <0.05 was considered significant.

Cell surface expression of viral antigens was evaluated by flow cytometric analysis. Briefly, cells were washed with PBS, incubated with specific MAbs preparations for 1 h at 4°C, and washed with PBS. Fluorescein-conjugated goat anti-mouse IgG was then added, and the mixture was incubated for 1 h at 4°C. Cells were then washed in PBS, fixed with 1% paraformaldehyde in PBS, and analyzed with a FACScan apparatus (Becton-Dickinson). For dual-color membrane immunofluorescence, cells were incubated with both 2L10-FITC and p11E8-RTIC for 1 h at 4°C and then washed with PBS. Cells were examined by fluorescence microscopy.

Western blotting. Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer, sonicated, boiled, and frozen until used. An amount approximating 3 × 10⁶ cells was loaded per lane of an 8% denaturing SDS-polyacrylamide gel as described by Laemmli (38). Proteins were transferred onto nitrocellulose membranes and processed for Western blotting (64). Immunoreactive proteins were visualized by chemiluminescence with the ECL detection kit (Amersham).

Southern blotting. Genomic DNAs were extracted from HHV-6-infected and mock-infected cells after 72 h of culture. Five micrograms of DNA was digested with HindIII and then electrophoresed through a 0.8% agarose gel. DNA was transferred onto nylon membranes and cross-linked with UV light. Membranes were blocked before being hybridized overnight with 32P-labelled pZVH14 as described previously (33). Mem-
RESULTS

Susceptibility of cell lines to HHV-6 infection. To evaluate whether HHV-6 can affect the expression of EBV antigens in B cells, it was first necessary to determine the susceptibility of different B-cell lines to HHV-6. All of the B-cell lines tested, with the exception of B95-8 cells, were found to be susceptible to infection with HHV-6. P3HR1, Akata, and Raji cells and LCL were easily infected and showed the characteristic CPE of HHV-6-infected cells. The susceptibility of these cell lines to HHV-6 infection was also monitored by immunofluorescence and Southern blotting. HHV-6 antigen expression in these various cell lines was detected by conventional immunofluorescence assays. The 41-kDa early component of HHV-6, which reacts with the D12 MAb, was detected in all of the infected cell lines, with the exception of B95-8 (data not shown). No reactivity of this MAb was observed immediately following infection (i.e., within 2 h), eliminating the possibility of false-positive results originating from the detection of antigens present in the inoculum. Late HHV-6 antigen expression was measured with MAb HAR1-5, which reacts with a glycoprotein of 110 and 60 kDa. Flow cytometric analyses performed on infected cell lines showed reactivity against all of the cell types, except B95-8. As shown in Fig. 1, 88% of HSB-2 cells were positive for HHV-6 gp110/60 at 72 h postinfection (p.i.) (positive control). Expression of HHV-6 gp110/60 on B-cell lines varied from 0% (B95-8) (data not shown) to 40% (Raji). Expression of HHV-6 antigens was also determined by using MAb 2D6, which reacts with a glycoprotein of 105 and 82 kDa. As shown in Fig. 2, no reactivity of the MAb was observed in mock-treated cells (left column) whereas cells positive for HHV-6 were easily observed at 72 h p.i. (right column). As shown, the reactivity of MAb 2D6 was restricted to the cytoplasm and the membrane of the infected cells. Most of the reactive cells showed CPE, shown by the enlarged size of the cells.

Southern blot experiments were also carried out to assess the presence of HHV-6 DNA following infection. Similar to the immunofluorescence data, all cell lines, except B95-8, were positive for HHV-6 DNA. HHV-6 probe pZVH14 corresponds to an 8.7-kb HindIII genomic sequence of HHV-6 strain GS. Digestion of cellular genomic DNA with HindIII should, if HHV-6 DNA is present, yield an 8.7-kb positive hybridization signal. As shown in Fig. 3, positive hybridization was observed in most of the cell lines. The strongest signals were observed for HSB-2-infected cells (positive control) and the P3HR1, Raji, and Akata cell lines.

Effect of HHV-6 on Zebra antigen expression. Having determined that HHV-6 can efficiently infect cells of the B lineage carrying the EBV genome, we studied the influence of HHV-6 infection on the expression of EBV lytic-cycle antigens. Figure 4 represents expression of the Zebra antigen following infection of various cell lines with HHV-6. Striking increases in Zebra antigen expression in the Raji (10-fold) and Akata (5-fold) cell lines were observed following HHV-6 infection. Zebra antigen expression in mock-treated Raji and Akata cells was <1%. Following infection of these cells with HHV-6, the percentages of cells expressing the Zebra antigen were 6% (Raji) and 4% (Akata). MAb BZ.1 showed no reactivity on HHV-6-infected EBV-negative cells. A typical CPE was observed following infection of these cell lines with HHV-6 (Fig. 4, inset). Enlarged, vacuolized cells with the tendency to form syncytia were noted. Infection of P3HR1 cells with HHV-6 upregulated Zebra expression by twofold. Seven percent of mock-treated P3HR1 cells were positive for Zebra, whereas about 15% of P3HR1 cells were positive for the same antigen following HHV-6 infection. B95-8 cells, which were not susceptible to HHV-6 infection, were used as a negative control. As shown in Fig. 4, treatment of these cells with HHV-6 had no effect on Zebra expression.
Effect of HHV-6 infection on expression of EBV early antigens. Having determined that HHV-6 can increase the expression of the immediate-early Zebra antigen in various B-cell lines, we focused our attention on the expression of other EBV EAs, i.e., EA-D and EA-R. We analyzed the expression of EA-D and EA-R in B-cell lines following infection with HHV-6. Expression of EA-D and EA-R at 48 h following treatment of cells with HHV-6 or mock infection fluid, is represented in Fig. 5. EA-D expression was increased by 10-fold in HHV-6-infected Raji cells (10% of EA-D-expressing cells) compared with that in uninfected cells (<1% of antigen-positive cells) (Fig. 5A). Similarly, a two- to fourfold increase in EA-D expression was noted following HHV-6 infection of P3HR1 (13%) and Akata (4%) cells compared with mock-treated P3HR1 (7%) and Akata (1%) cells (Fig. 5A). Analysis of EA-D by Western blotting (Fig. 5B) gave similar results. The EBV-negative BJA-B cell line served as a negative control. Expression of EA-D protein, following HHV-6 treatment, was increased in all of the cell lines tested. The P3HR1 and Raji cell lines and LCL showed an increase in both the 50- and 60-kDa components of the EA-D complex following infection by HHV-6. In Akata cells, only the 50-kDa component was found to be upregulated by HHV-6. Cell lines treated with 12-O-tetradecanoyl phorbol 13-acetate (TPA) or anti-human IgG (for Akata cells) served as positive controls; on average, 35% of TPA-treated cells and 32% of anti-IgG-treated Akata cells were positive for EA-D expression by immunofluorescence.

EBV EA-R expression in B-cell lines infected with HHV-6 was also studied. As shown in Fig. 5C, infection of Raji cells with HHV-6 led to an approximately 10-fold increase in EA-R-positive cells (10%), compared with mock-treated cells (<1%). Furthermore, 1.6- to 3-fold upregulation of EA-R

FIG. 2. HHV-6 antigen expression in fixed cells. Various cell lines were tested for susceptibility to HHV-6 infection. At 72 h p.i., cells were processed for immunofluorescence as described in Materials and Methods. Fixed cells were stained with MAb 2D6, which reacts against HHV-6 gp105/82. HSB-2 cells served as a positive control. Cells positive for HHV-6 were observed in the Raji, P3HR-1, and Akata cell lines.

FIG. 3. Southern blot analysis of HHV-6 DNA in cell lines. Cells were infected with HHV-6 for 72 h, after which genomic DNA was extracted, digested with HindIII, and electrophoresed through a 0.8% agarose gel. After transfer, DNA was hybridized with the HHV-6 pZVI14 probe. No signal was detected in sham-treated (−) cells. HSB-2 (positive control), P3HR1, Raji, and Akata cells and LCL were all positive for HHV-6 DNA following treatment with virus (+). No hybridization signal was detected in B95-8 cells. DNA molecular weight markers (phage λ cleaved with HindIII) are shown on the left (sizes are in kilobase pairs).

FIG. 4. Zebra antigen expression following HHV-6 infection of B-cell lines. Cells were either mock infected or HHV-6 treated for 48 h, after which they were analyzed for EBV Zebra antigen expression by immunofluorescence with MAb BZ.1 as described in Materials and Methods. The percentage of Zebra-expressing cells is shown on the y axis. *P < 0.05. The insets illustrate typical CPE, with large, refractile cells in HHV-6-infected Raji (left) and Akata (right) cells. No morphological evidence of HHV-6 infection was detected in B95-8 cells.
protein expression was observed in HHV-6-infected P3HR1 and Akata cells (Fig. 5C).

The kinetics of EA-D induction by HHV-6 in P3HR1 and Raji cells was also studied. As shown in Fig. 6, expression of EA-D in P3HR1 and Raji cells was both time and virus concentration dependent. At 24 h p.i., expression of EA-D in both cell lines was similar to that recorded for mock-treated cells. At 48 h p.i., an increase in EA-D was detected in both P3HR1 and Raji cells. When cells were infected with 10-fold less virus, EA-D induction was delayed by 24 h (data not shown). Finally, by 72 h p.i., maximal induction of EA-D was observed in both cell lines following infection with 10^4 50% tissue culture infective doses of HHV-6.

**Influence of HHV-6 on expression of EBV late antigens.**

EBV lytic antigens are expressed in a cascade fashion: first, the immediate-early genes, the early genes, and then the late genes are expressed. This study has thus far focused on the expression of immediate-early and early genes of EBV. To analyze whether HHV-6 can also influence the expression of EBV late genes, we studied the expression of two structural proteins of EBV following infection of B cells with HHV-6. First, as shown in Fig. 7, HHV-6 upregulated the synthesis of the VCA in the P3HR1 and Akata cell lines. Expression of the VCA in mock-treated P3HR1 and Akata cells was evaluated at 6 and 3%, respectively. Following 72 h of infection with HHV-6, the number of VCA-positive cells was increased by two- to threefold. Thirteen percent of HHV-6-infected P3HR1 cells expressed the VCA, while 12% of infected Akata cells were positive for the VCA. The MAb used reacted with the 125-kDa component of the EBV VCA. Raji cells, which are known not to synthesize late antigens, were used as a negative control.
The second structural protein of EBV that was studied following infection of B cells with HHV-6 was major envelope glycoprotein gp350. Cell surface expression of EBV gp350 was assessed by flow cytometry with MAb 2L10 and 72A1. As shown in Table 1, expression of gp350 was induced by HHV-6 in both the Akata and P3HR1 cell lines. Expression of gp350 in mock-treated cells between 3 and 8% was recorded, depending on the MAb used. By comparison, up to 20 and 24% of HHV-6-infected P3HR1 and Akata cells expressed the gp350 antigen. A two- to threefold increase in gp350 antigen-positive cells was observed, irrespective of the MAb used. It is noteworthy that the percentage of gp350-expressing cells was higher than that of VCA-expressing cells. It is unclear whether this is due to the difference in sensitivity of the two assays used. Treatment of Akata cells with anti-human IgG or P3HR1 with TPA (positive controls) increased gp350 expression by 5- to 10-fold. Expression of gp350 was also studied with Raji cells. As shown in Table 1, mock-, HHV-6-, or TPA-treated cells failed to express the gp350 molecule.

**TABLE 1.** Expression of EBV gp350 on cell lines following infection with HHV-6.

<table>
<thead>
<tr>
<th>Cell line and treatment</th>
<th>% of cells expressing gp350</th>
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<tbody>
<tr>
<td></td>
<td>MAb 2L10</td>
</tr>
<tr>
<td>Akata</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>3</td>
</tr>
<tr>
<td>HHV-6</td>
<td>18</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>44</td>
</tr>
<tr>
<td>P3HR1</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>3</td>
</tr>
<tr>
<td>HHV-6</td>
<td>11</td>
</tr>
<tr>
<td>TPA</td>
<td>37</td>
</tr>
<tr>
<td>Raji</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>0</td>
</tr>
<tr>
<td>HHV-6</td>
<td>0</td>
</tr>
<tr>
<td>TPA</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were treated with either mock-infection fluid, HHV-6, anti-IgG, or TPA for 72 h. Cells were washed with PBS, incubated with a MAb (2L10 or 72A1) for 1 h at 4°C, and washed with PBS. Fluorescein-conjugated goat anti-mouse IgG was then added, and the mixture was incubated for 1 h at 4°C. Cells were washed in PBS, fixed with 1% paraformaldehyde, and analyzed with a FACScan apparatus (Becton-Dickinson). Results are expressed as percentages of gp350 antigen-expressing cells after scoring of 10,000 events.

**TABLE 2.** Effects of infectious and inactivated HHV-6 on induction of Zebra and EBV EA-D.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean % of antigen-positive Raji cells ± SD</th>
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<tbody>
<tr>
<td>HHV-6 gp110/60</td>
<td>Zebra</td>
</tr>
<tr>
<td>Mock infection</td>
<td>0</td>
</tr>
<tr>
<td>HHV-6</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>UV-irradiated HHV-6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Heat-inactivated HHV-6</td>
<td>&lt;1</td>
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</table>

* Raji cells were treated with either mock-infection fluid, infectious HHV-6, UV-irradiated HHV-6, or heat-inactivated HHV-6 for 48 h and then washed and processed for immunofluorescence as described in Materials and Methods. The values shown are means of three different experiments. HHV-6 gp110/60 was analyzed with MAb HAR 1-5, Zebra was analyzed with MAb BZ 1, and EA-D was analyzed with MAb R3.

This is due to the difference in sensitivity of the two assays used. Treatment of Akata cells with anti-human IgG or P3HR1 with TPA (positive controls) increased gp350 expression by 5- to 10-fold. Expression of gp350 was also studied with Raji cells. As shown in Table 1, mock-, HHV-6-, or TPA-treated cells failed to express the gp350 molecule.

**DISCUSSION**

Latency of herpesviruses is a very complex phenomenon involving intricate gene regulation. Each herpesvirus appears to have evolved in a different way in order to reside, undetected, in its natural host. The potential to remain latent, with occasional reactivation, has contributed to the widespread prevalence of these viruses.

EBV ensures its survival by infecting two very distinct cellular counterparts. (i) the virus productively infects epithelial cells of the oropharynx; dissemination occurs through shedding of virions in saliva (17). (ii) EBV nonproductively...
infected primary B lymphocytes, resulting in life-long latent infection. In vitro, EBV gene expression in B cells is restricted to six EBV nuclear antigens, three membrane proteins (LMP1, LMP2A, and LMP2B), and two small untranslated RNAs called EBV-encoded RNAs (EBER1 and EBER2) (12, 13).

The EBV lytic cycle has been most extensively studied by reactivating latently infected B cells with various chemical inducers (30, 43, 72). The most frequently utilized inducer is the tumor-promoting agent TPA. Although studies with chemical inducers of EBV replication have been relatively well documented, those dealing with viruses as stimuli of EBV replication are limited. Such studies have focused on EBV reactivation following superinfection of the Raji B-cell line with a defective EBV strain from the P3HR1 cell line (5, 56). Raji is an EBV genome-positive Burkitt tumor cell line that has at least two viral genomic deletions, limiting the expression of EBV to EAs (23, 52). However, expression of late genes can be induced through complementation after superinfection with the P3HR1 virus (5, 54). The need to identify other agents that trigger EBV replication is of medical importance, especially in regard to the mechanism and etiology of EBV-associated disease processes. In the present study, we provided evidence indicating that HHV-6 induces EBV to reactivate in B-cell lines.

HHV-6 infection of B-cell lines, namely, Raji, Akata, and P3HR1, resulted in an increase of Zebra protein synthesis. Zebra is a 38-kDa nuclear protein, encoded by the BZLF1 genomic segment, whose function is to transactivate downstream, early EBV genes by binding to consensus DNA sequences (9, 11, 34, 61). This immediate-early EBV protein is known to be crucial for the switch from latency to the lytic cycle (11, 34, 61). It can be tentatively hypothesized that HHV-6 reactivates EBV in B cells by inducing the immediate-early transactivator Zebra. Zebra activation would then lead, through a cascade effect, to the expression of other lytic antigens of the EBV cycle. Whether Zebra expression is triggered directly by transactivating proteins of HHV-6 or involves the participation of cellular factors is still speculative. However, the need for infectious HHV-6 for Zebra activation suggests that the virus does not carry a transactivating protein capable of such an effect. Results indicate that the viral genetic information that encodes such an activator is destroyed by UV irradiation and therefore can no longer encode a functional protein. The only B-cell line tested that showed no such increase of EBV proteins following treatment with HHV-6 is the B95-8 cell line. As demonstrated by Southern blotting and immunofluorescence, no evidence of HHV-6 infection was recorded. A likely explanation for the inability of HHV-6 to infect B95-8 cells is that HHV-6 has a narrow host range and the B95-8 cell line is derived from marmoset peripheral blood lymphocytes infected with EBV from a patient with infectious mononucleosis. This corroborates the results of Lusso et al. (46), who showed that of six monkey species tested, HHV-6 was able to infect only peripheral blood leukocytes derived from chimpanzee. Included in the study were peripheral blood leukocytes from a common marmoset, which were found to be refractory to HHV-6 infection (46). The lack of a cellular receptor for HHV-6 on the surface of B95-8 cells is one hypothesis that explains the above-described results. Susceptibility of B cells to HHV-6 infection seems, therefore, to be a requirement for the observed EBV antigen induction.

Similar to induction of the Zebra antigen, other early EBV antigens were also upregulated by infection of HHV-6. Infec-
tion of B cells led to an increase in the D component, which consists of two proteins encoded by the BMLF1 (~50 kDa) and BMLF1 (~60 kDa) genomic segments (65, 66). The EA-D proteins are involved in the transactivation of other EBV genes (41, 65, 66). Furthermore, HHV-6 infection of B cells resulted in upregulation of the 85-kDa protein of the R component, encoded by the BORF2 gene segment of EBV (18, 44). The 85-kDa protein of EBV shares extensive sequence homology with the ribonucleotide reductase of herpes simplex virus (18). The EA-D and EA-R components can be distinguished on the basis of their differential solubilities in methanol (26). Subsequent to the synthesis of the EAs is the replication of viral DNA, followed by synthesis of structural proteins, also termed late antigens. These proteins are the last to be synthesized prior to viral assembly and liberation. Analysis of HHV-6-infected B-cell lines also demonstrated an increase in the

FIG. 8. P3HR-1 cells expressing both gp350 of EBV and gp56/20 of HHV-6. P3HR-1 cells were infected with HHV-6 for 72 h and tested for expression of gp350 of EBV and gp56/20 of HHV-6 by dual-color membrane immunofluorescence. MAb 2L10, which reacts with gp350 of EBV, was labelled with FITC, and MAb P11E8, directed against gp56/20 of HHV-6, was labelled with RTIC. Cells were incubated with both MAbS for 1 h at 4°C and then washed with PBS. Cells were fixed with 1% paraformaldehyde in PBS before being observed by fluorescence microscopy. The left column represent cells examined for 2L10-FITC reactivity, whereas the right column represent the same cells observed for P11E8-RITC reactivity. The first row represents mock-infected P3HR-1 cells stained with both MAbS. The next three rows represent different fields of cells expressing both antigens.
synthesis of late EBV antigens in at least two cell lines. Both the VCA and envelope glycoprotein gp350 showed increased expression after HHV-6 infection in the P3HR1 and Akata cell lines, suggesting that HHV-6 can induce the full EBV replicative cycle. In addition, results obtained following dual-color membrane immunofluorescence staining of HHV-6-infected P3HR1 cells indicate that the B cells that replicate HHV-6 are also those that replicate EBV. Infection of Raji cells with HHV-6 resulted in no detectable gp350 or VCA suggesting, among other things, a lack of functional or genomic complementation between these two viruses. This could well be related to the limited degree of homology between EBV and HHV-6 DNAs (39).

Inducers of EBV replication, such as HHV-6, are of importance when considering serious complications associated with recurrent EBV infections. In patients afflicted with genetic (X-linked or acquired (HSV or immunosuppressive therapy) immunodeficiencies, EBV infection and reactivation can lead to grave and sometime fatal outcomes (36, 53, 70, 71). Because of reduced or nonfunctional immunity, B-cell proliferation (possibly through immortalization of these cells by reactivated EBV) is observed. EBV-associated polymorphic B-cell lymphoma is one possible outcome of such lymphoproliferation (32, 37). HHV-6 has also been linked to lymphoproliferative disease and therefore may also contribute to lymphomagenesis (27). HHV-6 infection is one of the major factors that lead to B-cell proliferation in certain cases. HHV-6 reactivation of HHV-6 in immunocompromised patients may further accentuate EBV replication and thereby contribute to the lymphoproliferative observed.

On the basis of immunoglobulin gene rearrangement studies or EBV terminus analysis, the tumors found in patients are, at least initially, polyclonal in origin (7, 10). This suggests that neoplastic cells arise from the initial infection of many different B lymphocytes by EBV. With time, the lymphoma evolved to an oligo- or monoclonal state (7, 10). By inducing EBV, HHV-6 may, therefore, contribute to an increase in the B-cell pool infected with EBV, thereby increasing the chances of developing an EBV-related lymphoma. Taken together, the present study illustrates the interactions between two viruses, both belonging to the same family, which can infect B lymphocytes. We have demonstrated that infection of EBV genomically positive cells by HHV-6 leads to reactivation of EBV. Further studies are needed to better understand how viral cooperation may contribute to disease processes.

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