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Human Herpesvirus 6 Variant A, but Not Variant B, Infects EBV-Positive B Lymphoid Cells, Activating the Latent EBV Genome through a BZLF-1-Dependent Mechanism

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ABSTRACT

Human herpesvirus 6, a predominantly T lymphotropic virus, has been recently shown to infect some EBV-positive B cell lines, and to induce in them the activation of the EBV lytic cycle. Here we have confirmed and extended such observations, showing that (1) this phenomenon is restricted to the variant A of HHV-6: in fact two isolates belonging to the HHV-6 variant B (BA92 and Z29) were neither able to infect any B cell line, independently of the EBV status, nor to induce the EBV genome expression. The only exception is represented by the P3HR1 cells, in which, however, the infection by the variant B does not determine induction of EBV antigens; (2) the presence of the EBV genome contributes to the susceptibility of the B cell lines to HHV-6 infection, increasing the binding sites and the percentage of infectable cells, as detected by immunoelectron microscopy; and (3) HHV-6 infected T cells, transfected with plasmids bearing the promoter regions of the EBV early genes BZLF1 and BMRF1, show a strong transactivation of these promoters.

INTRODUCTION

Human herpesvirus 6 (HHV-6) is the etiologic agent of exanthem subitum and, like Epstein–Barr virus (EBV), is present in a high percentage of the healthy population.[1–4] A large number of HHV-6 isolates have been derived worldwide, and have been subgrouped in two variants: variant A (whose prototype is the initial isolate GS) and variant B, with characteristics similar to the Z29 prototype. Although variant A strains are prevalently isolated from AIDS and all the isolates from exanthem subitum belong to variant B, the distinction between the two variants is mainly based on biological properties, such as host cell tropism, antigenicity, and genomic polymorphism.[5–8] Studies *in vitro* show a close interaction of HHV-6 with the human immunodeficiency virus (HIV-1). The dual infection of $CD4^+$ cells, the induction of CD4 by HHV-6, and the molecular crosstalk among the two viruses strongly suggest HHV-6 as a potential cofactor in the pathogenesis and progression of AIDS.[9–13] HHV-6 may also interact *in vivo* with EBV, which is also known to be involved in AIDS progression or AIDS-associated lymphoproliferative diseases.[14,15] Preliminary observations were initially reported by some of us on the activation of EBV lytic cycle by HHV-6 infection,[16] and a more extensive study has been recently published by Flamand *et al.*[17]

With the present study we describe the infectability by HHV-6 of a panel of lymphoid cell lines, provided they are EBV genome positive. We show that only variant A HHV-6, but not variant B, activates the resident EBV genome, and we also offer a molecular analysis of the mechanisms involved in the activation of the EBV latency *in vitro*.

MATERIALS AND METHODS

Cells and viruses

All the cell lines were grown in RPMI 1640 medium supplemented with 10% FCS plus antibiotics. The GS and U1102 strains (variant A) and BA92 and Z29 strains (variant B) of HHV-6 were employed in this investigation. The GS strain was propagated in the immature T cell line HSB-2 as described elsewhere,[18] the U1102 isolate was grown in J-Jhan cells, and the BA92[8] and Z29 isolates were grown in PHA-activated human cord blood mononuclear cells.

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Infection procedure and immunofluorescence

For infection, 5 × 10^6 pelletted cells were incubated with 1 ml supernatant of a 7-day infected culture. After 4 hr at 37°C the cells were washed once and resuspended in complete medium. When cytopathic effect was visible, the cells were tested for the presence of viral antigens by indirect immuofluorescence. The following monoclonal antibodies (MAbs) were used: anti-HHV-6 p41/38 (Abi, Columbia, MD), R3 and L2 (directed against EBV early and capsid antigens, respectively, gift of G. Pearson), and a rabbit polyclonal antibody (gift of G. Miller) that detects the BZLF-1 immediate early protein ZEBRA.

For the experiments described in Table 4, HHV-6 was allowed to adsorbe to the cells at 4°C for 4 hr in the absence of MAb. The cells were centrifuged to remove unadsorbed virus and rinsed twice by centrifugation. MAb 2E4 (gift of G. Campadelli-Fiume), which has been shown to inhibit penetration of HHV-6 in susceptible cells,[19] was then added and allowed to bind to cells carrying attached virus for 3 hr at 4°C. Cells were then incubated at 37°C in complete medium. For heat inactivation the HHV-6 stock was incubated for 60 min in water bath at 56°C. For UV inactivation the virus was directly exposed to the UV light source, receiving a dose of 230 mW/cm^2 for 5 min.

Immunoelectron microscopy

The cells were washed, pelleted, resuspended in 2 ml of stock virus (titer 10^5 $TCID_{50}$), and incubated for 1 hr at 4°C, washed twice in cold phosphate-buffered saline, and fixed in 0.5% glutaraldehyde for 1 hr at 25°C in the same buffer. For immunolabeling the cells were incubated with a high titered HHV-6 human serum, washed extensively, and labeled for 2 hr at 4°C with colloidal gold conjugated with protein A, as previously described.[18] Immunolabeled cells were postfixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated in acetone, and embedded in Epon 812.

Transfection and CAT assay

Promoter regions from BZLF1 (nucleotides from position −265 to −29) and BMRF1 (nucleotides from position −331 to +1) were cloned upstream the CAT gene. Thirty micrograms of reporter CAT construct ZpCAT, EABSCAT, or promoterless E4CAT was transfected by electroporation in 10^7 HSB-2 cells either uninfected or 5 days after infection with HHV-6-GS. CAT activity was assayed 2 days after transfection as described.[20]

RESULTS AND DISCUSSION

In an initial series of experiments a number of EBV genome-positive and -negative B cell lines were screened for their susceptibility to infection by either variant A or B of HHV-6. Infection was recognized by the appearance of cells showing the characteristic cytopathic enlargement and by the expression of HHV-6-specific antigens. The EBV genome-positive Raji, P3HR1, Rael, Akata, IARC 171, E95A-BL41, and E95A-BL28 were all infected by HHV-6 variant A (Table 1). Percentage of infected cells peaked at day 4 from viral exposure. All the EBV genome-negative tested BJAB, DG75, BL28, and BL41 and the marmoset EBV genome-positive cell line B95-8 were not infected by HHV-6. BL28 and BL41 became susceptible to HHV-6 infection after they were converted to EBV genome positivity by *in vitro* EBV infection with the B95-8 strain (E95A-BL28 and E95A-BL41, respectively). With the exception of P3HR1 cells, both variant B isolates (BA92 and Z29) could never infect any of the cell lines, independently of their EBV status. EBV may therefore induce or up-regulate a specific receptor for HHV-6 variant A. Since the membrane receptor for HHV-6 has not been yet identified, to verify such an hypothesis we attempted to visualize viral binding by immunoelectron microscopy. Other approaches to measure viral binding by indirect immunological detection with fluorescent reagents and FACScan analysis or using radiolabeled virus with a procedure similar to that used by Miller and Hutt-Fletcher for EBV[21] did not allow us to detect signals above background (not shown), probably due to the low number of receptors. By immunoelectron microscopy, at 4°C very few viral particles attached to the plasma membrane of the EBV genome-negative BL41 and BL28 cells were visible and immunolabeled. After their EBV conversion, the number of attached virions doubled (Fig. 1) (on average approx. 2 virions/cell section), and the percentage of cells with surface-bound viral particles increased by 4-fold. (Table 2). We estimated the total number of virions bound to a single cell to be approximately 80, 160, and 300 for EBV-negative BL41 cells, EBV-converted BL41 cells, and HSB-2 cells, respectively. Thus, EBV-infected cells have enhanced susceptibility to HHV-6 infection, possibly due to either *de novo* induction or up-regulation of the yet unidentified HHV-6 receptor. In this regard, of interest is a recent observation by Birkenbach *et al.*,[22] which identified in the EBV-converted BL41 cells two novel lymphocyte-specific G protein-coupled peptide receptors that were absent in the EBV-negative counterpart. That the number of virus binding sites is a major determinant restricting the host range of human B cell lines has also been recently shown for the B lymphotropic papovavirus infection.[23] The trans-acting EBNA-1 may have a role in driving the expression of the HHV-6 receptor, since it is the only EB viral product translated in Rael and Akata cells.

TABLE 1. INFECTION OF B CELL LINES BY HUMAN HERPESVIRUS 6[a]

Cell line	Presence of EBV genome	HHV-6 variant A		HHV-6 variant B	
		GS	*U1102*	*BA92*	*Z29*
Raji	+	+	+	−	−
P3HR1	+	+	+	+	±
Rael[b]	+	+	n.d.	−	n.d.
B95-8	+	−	n.d.	−	n.d.
Akata[b]	+	+	+	−	n.d.
IARC 171	+	+	n.d.	−	n.d.
BJAB	−	−	n.d.	n.d.	n.d.
DG75	−	−	−	−	n.d.
BL41	−	−	−	−	−
BL28	−	−	n.d.	−	−
E95A-BL41	+	+	n.d.	−	n.d.
E95A-BL28	+	+	n.d.	n.d.	n.d.

[a]Five experiments were performed for each cell line.
[b]Rael and Akata cells express only EBNA-1.

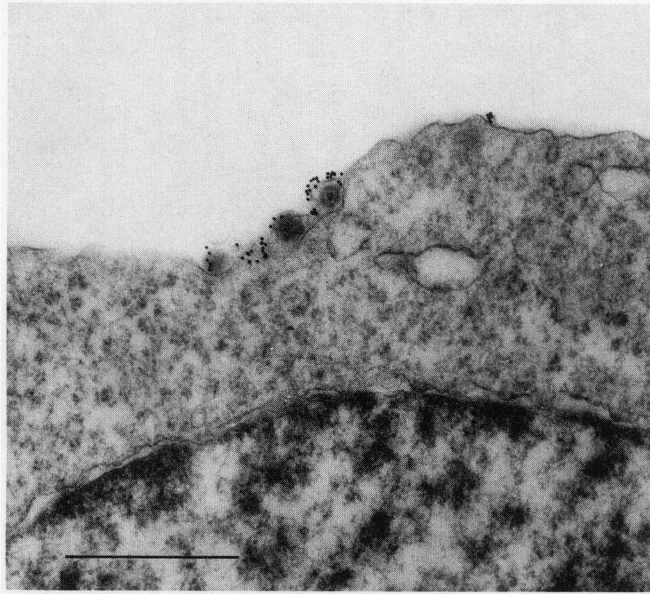

FIG. 1. Immunoelectron microscopic labeling of HHV-6 over the plasma membrane of EBV-converted BL41 cells. Few viral particles attached to the cell surface are visible and immunolabeled (bar = 1 μm).

Alternatively, HHV-6 infection could be modulated by the activation of receptor molecules already present on the surface. For instance, cell adhesion molecules increase their avidity after stimuli varying the levels of intracellular calcium.[24] We next investigated the effect of HHV-6 infection on the induction of the EBV lytic cycle in several of the EB viral genome-positive cell lines tested (Table 3). Raji and Rael are derived from EBV-positive Burkitt lymphomas: the former is induceable by several stimuli, but the infection is abortive and limited to the expression of EBV early antigens; the latter is not permissive to the EBV lytic cycle. P3HR1 also is tumor derived and is exceptionally characterized by the constitutive expression of EBV lytic antigens in a limited fraction of cells. IARC 171 is a lymphoblastoid cell line derived upon *in vitro* immortalization of adult B lymphocytes from peripheral blood. Within this type, a variable number of cells are usually permissive to EBV. Exposure to HHV-6 significantly increased the proportion of EBV antigen-positive cells (Table 3). In Raji, EBV induction was limited to early antigen expression, as usually observed with other promoters of the viral cycle. Notably, in P3HR1 only HHV-6 variant A, but not variant B, seems to have an inducing effect on the EBV lytic cycle, although modest.

Inactivating the viral inoculum by UV light or heat abrogated the EBV induction (Table 4). Treatment with a monoclonal antibody (2E4) shown to inhibit HHV-6 entry but not the binding to the cell,[19] also resulted in blocking of the inducing

TABLE 2. QUANTITATION OF SURFACE BOUND HHV-6 ON BL41 AND E95A-BL41 CELLS, AS DETECTED BY IMMUNOELECTRON MICROSCOPY

Treatment	*Number of cells examined*	*Number of virions per cell section ± SEM*	*% of cells with bound virions*
BL41/HHV-6	74	1 ± 0.2[a]	6.7
E95A-BL41/HHV-6	67	2.04 ± 0.3[a]	31
HSB-2/HHV-6	70	3.8 ± 0.4	30

[a]Statistically significant with $p < 0.01$.

TABLE 3. INDUCTION OF VIRAL ANTIGENS IN EBV-POSITIVE CELLS INFECTED BY HHV-6[a]

Cell line	*HHV-6 p41*	*EBV-EA*	*EBV-VCA*	*ZEBRA*
		(percentage of antigen	*expressing cells)*	
Raji	Neg	Neg	Neg	Neg
Raji + HHV-6 (GS)	4	2	Neg	2
Raji + HHV-6 (U1102)	3	2	Neg	n.d.
Raji + HHV-6 (BA92)	Neg	Neg	Neg	Neg
Raji + HHV-6 (Z29)	Neg	Neg	Neg	Neg
Rael	Neg	Neg	Neg	Neg
Rael + GS	3	2	0.1	1
P3HR1	Neg	3.5[a]	3.6[b]	3[c]
P3HR1 + GS	6	8[a]	7[b]	7[c]
P3HR1 + U1102	2	6	4	n.d.
P3HR1 + BA92	7	4	3.8	3
P3HR1 + Z29	<1	3	4	n.d.
IARC 171	Neg	0.1[d]	0.1[e]	Neg
IARC 171 + GS	4	2.5[d]	2.5[e]	n.d.

[a]One experiment out of five. (a vs a, b vs b, c vs c, d vs d, e vs e = $p < 0.001$ as calculated by the Chi square test.)

TABLE 4. EFFECT OF VIRUS INACTIVATION OR TREATMENT WITH 2E4 MAb ON THE HHV-6 INFECTION AND ON ITS EA-INDUCING ACTIVITY IN RAJI CELLS[a]

Cells and treatment	*HHV-6 p41*	*EBV-EA*
HSB-2 + GS	40%	Neg
HSB-2 + GS + 2E4	10%	Neg
HSB-2 + GS (UV inact.)	Neg	Neg
HSB-2 + GS (heat inact.)	Neg	Neg
Raji + GS	3%	3%
Raji + GS + 2E4	Neg	Neg
Raji + GS (UV inact.)	Neg	1%
Raji + GS (heat inact)	Neg	Neg

[a]One experiment out of three.

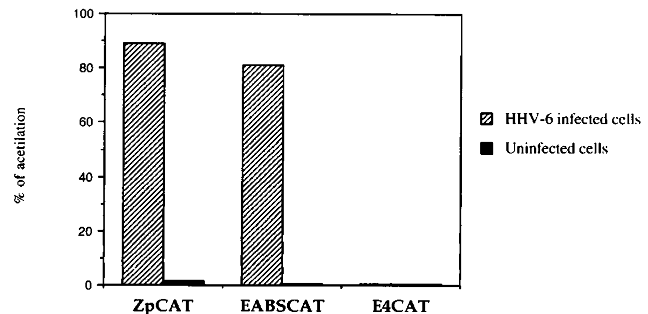

FIG. 2. Activation of CAT reporter genes in HHV-6-infected or -uninfected HSB-2 cells.

activity (Table 3). Active HHV-6 viral replication seems, therefore, to be required for reactivating the EBV latent genome. To attempt to define whether the EBV-inducing effect is due to early or late gene products of HHV-6, Raji cells were infected with HHV-6 (GS) in the presence or absence of phosphonoacetic acid (PPA), which is known to inhibit viral DNA polymerase. No changes in the percentage of EBV EA-positive cells were observed after PAA treatment (0.8 mM), suggesting that the inducing effect was due to an early HHV-6 gene product. Control experiments using B95-8 cells showed a full inhibition of EBV VCA expression (data not shown).

Recently, Flamand *et al.*[17] described a similar induction of EBV antigens expression by HHV-6 variant A. Lytic antigens coexpression by the two viruses was also observed within P3HR1 cells. When we examined whether HHV-6 and EBV antigens were coexpressed in Raji, double fluorescence microscopy experiments never confirmed coexpression of the HHV-6 p41 with either EA or ZEBRA early proteins of EBV (not shown). It is possible that this discrepancy is due to the use of different antibody reagents or cell lines. In fact, in the study of Flamand *et al.* HHV-6 may have infected those P3HR1 cells that were already actively replicating EBV, since a significant percentage of EBV-EA and VCA-positive cells is normally present in the P3HR1 cell line. Here, the lack of cells involved in a productive dual infection would suggest to us that EBV activation is not directly achieved through the molecular interaction of the two viral genomes or of their products, but it could be mediated by cytokines released by the HHV-6-infected cells,[25] as in the case of human immunodeficiency virus reactivation,[26] although several attempts to induce the EBV replicative cycle by the addition of UV-inactivated supernatant of HHV-6 infected Raji cells failed to give a positive result.

We finally focused our attention on the hypothetical target(s) of HHV-6 activation mechanisms on EBV at the molecular level. The disruption of EBV latency is a phenomenon that occurs spontaneously in a very low percentage of cells, and can be induced by a variety of chemical and physical stimuli.[27] We reported earlier that calcium modulation can lead to the activation of the EBV genome, and that this phenomenon may be a consequence of the activation of cellular protein kinase C.[28,29] More recently it has been discovered that initiation of the EBV lytic cycle is dependent on the synthesis of the BZLF-1 gene product, ZEBRA.[30,31] Thus, to directly assess whether BZLF-1 synthesis is also involved in the induction of EBV antigens during HHV-6 infection, we infected Raji cells with HHV-6 and after a 3-day incubation immunofluorescence was performed to detect ZEBRA using an anti-ZEBRA rabbit polyclonal antibody.[32] As shown in Table 2, approximately 2% of ZEBRA positive cells were detected. Northern blot analysis revealed at 4 hr after infection an increase in BZLF-1 mRNA expression of 3- to 4-fold over background, as determined by densitometric analysis. BZLF-1 mRNA induction lasted for 72 hr, afterward decreasing to background level (data not shown). The activation of both BZLF-1 and BMRF-1 genes was also analyzed in an EBV-negative environment. CAT assays were performed after transfection of plasmids ZpCAT and EABSCAT in the EBV genome-negative immature T cell line HSB-2 previously infected by HHV-6 variant A. These plasmids drive the expression of the reporter CAT gene under the control of BZLF-1 and BMRF-1 promoters, respectively. As shown in Figure 2, transfection of the HHV-6-infected HSB-2 cells resulted in a strong acetylation pattern (89 and 81% for ZpCAT and EABSCAT, respectively), compared to mock-infected cells (0.7 and 1.5%). These results suggest an independent transactivation of the two genes following HHV-6 infection. The levels of ZEBRA expression in HHV-6-infected Raji cells can be detected either by IF or by Northern blot and do not seem to be proportional to the levels of transactivation of its promoter achieved in the HHV-6-infected HSB-2 cells, as detected by CAT assay. Some hypotheses can be made to explain these data: (1) HSB-2 cells are much more susceptible to HHV-6 infection than B cells; (2) the absence of EBV DNA sequences positioned upstream of the promoter region cloned in ZpCAT, known to be bound by repressors,[33] might have removed some control mechanisms active in the genomic context; (3) differences in the intracellular environment for the presence of cell-specific factors could account for the tight control in the expression of ZEBRA in B cells versus the enhanced BZLF-1 promoter activation in T cells.

To evaluate whether a similar interaction between HHV-6 and EBV may occur also *in vivo*, thus potentially contributing to the etiopathogenesis of some of the EBV-associated diseases, it will be of interest to investigate if EBV reactivation occurs in individuals with active infection by HHV-6.

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