Human herpesvirus-6B (HHV-6B), a causative agent of exanthem subitum, infects human adult T cell leukemia (ATL) cell lines. We established a persistent HHV-6B infection in an ATL cell line, TaY, in the presence of 20 units/ml interleukin-2 (IL-2). The HHV-6B infected culture proliferated with a constant ratio of infected (1%) to the uninfected (99%) cells. When the IL-2 concentration was reduced to 5 units/ml, the number of infected cells in the culture increased transiently by 60% in 11 days, a new balance of 25% infected cells and 75% uninfected cells was established thereafter. PCR analysis confirmed a 125-fold increase in the amount of viral genome in the culture, while the treatment with ganciclovir reduced the proportion of infected cells, indicating that an efficient replication of virus was induced in the culture. Both of these cultures were maintained in the presence of 20 or 5 units/ml IL-2 over one year without loss of infected cells. Interestingly, we found that cultures containing the infected cells grew significantly faster than the parental uninfected cells at the same concentration of IL-2. The infected culture continued to grow for 7 days even in the absence of IL-2. Because the infection induces cell cycle arrest, these results indicate that the HHV-6B-infected ATL cells stimulate the growth of the uninfected cells during persistent infection in culture.

Key words: HHV-6, ATL, IL-2

Introduction

Human herpesvirus 6 (HHV-6) is a betaherpesvirus first isolated from immunocompromised patients with a lymphoproliferative disorder1. Two subgroups, HHV-6A and HHV-6B, have been defined based on distinctive genomic, antigenic, and biological properties2-4. HHV-6B is detected in the peripheral blood, saliva, and lymph nodes of healthy individuals, whereas HHV-6A is found less frequently and shows a greater neurotropism than HHV-6B5. HHV-6B is a causative agent of the childhood disease exanthem subitum6, while little is known about the diseases related to HHV-6A. HHV-6 is also associated with rash, graft-versus-host disease, and pneumonitis in bone marrow transplant recipients. Primary infection of HHV-6 occurs within the first 2 years of life and is followed by lifelong persistence in the host7. HHV-6 infects primarily CD4+ T cells5,10. HHV-6 also established latency in a monocyte lineage of cells following primary infection11, and expressed latency-associated transcripts of immediate-early 1 and 2 genes12.

Since HHV-6 and human immunodeficiency virus (HIV) show a similar tropism for CD4+ T cells and are found in AIDS patients, the interaction between HHV-6 and HIV has been intensely studied. HHV-6 and HIV coinfect CD4+ T cells in a synergistic manner in vitro13,14. Although HHV-6 can transactivate the HIV pro
HHV-6 infection activates the expression of CCR5 in T cells. HHV-6 increases the expression of interleukin-18, the IL-2 receptor, and members of the tumor necrosis factor \( \alpha \) superfamily receptors, while down-regulating the expression of type 2 cytokines including IL-10, IL-10 receptor, and IL-14. Thus, HHV-6 infection drives T cells to a type 1 immune response that is related to the development of associated diseases. Furthermore, HHV-6 modulates the expression of chemokines and their receptors. The expression of RANTES is upregulated as described above, and that of CXCR4 is suppressed. HHV-6 expresses its own proteins that mimic chemokines and receptors. The open reading frame U83 encodes a CC chemokine, and both U12 and U51 encode chemokine receptors.

Although the significance of HHV-6 in HIV-1 infection is well recognized, little is known about the interaction between HHV-6 and human T lymphotrophic virus 1 (HTLV-1), which has a similar CD4 T cell tropism. About 5% of HTLV-1-infected individuals develop adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) after a long latent period, although the majority of seropositive individuals are asymptomatic carriers. It has been demonstrated in vitro that HHV-6 can productively infect several ATL cell lines. Recently we have established a persistent HHV-6B infection in an ATL patient. HTLV-1 genome harbors in TaY cells and expresses p24 protein. HHV-6B infection does not affected a level of p24 expression. The ratio of HHV-6B-infected to uninfected cells in the culture achieved a balance at 1:100, and the infected cells have not been lost from the culture over a period of one year, suggesting possible interactions between HHV-6B and ATL cells.
s), and extension (72°C for 1 min); and finally extension (72°C for 2 min). The reaction was performed in a mixture (50 μl) containing 1 pmol/μl of each primer, total DNA and the Expand High FidelityPLUS PCR System. The amount of total DNA used for the reaction is shown in the figures. The PCR products were examined by electrophoresis using a 1.5% agarose gel.

Ganciclovir sensitivity

Cells were plated in a 24-well plate (2 x 10^5 cells/well) and cultured for 6 days in the presence of 12 μM ganciclovir. As controls, cells were treated similarly with 10 μM interferon-γ or DMSO. The culture medium was replaced with fresh medium containing the same drugs after 4 days.

Cell growth profile analysis

Cells were washed once with PBS and plated in a 24-well dish. Each well contained 2 x 10^5 cells in 1 ml of culture medium containing IL-2 as indicated in the figure. Living cells were counted at 3, 7 and 10 days after plating. Cells were diluted 3-fold at 3 and 7 days. Multiple wells were prepared for each cell type. To examine the cell growth in the absence of IL-2, the cells maintained in 20 units/ml IL-2 were washed twice with PBS and transferred to the medium without IL-2. Living cells were counted similarly.

Results

Interleukin-2 (IL-2) regulated persistent HHV-6B infection of an ATL cell line. We infected the ATL cell line TaY with HHV-6B (strain OK) in the presence of 20 units/ml IL-2. The infected cells showed cytopathic effects of the virus and produced infectious virions within 5 days of infection as a typical course of betaherpesvirus infection. However, we found that viral replication did not overwhelm cell growth in this culture, and the cells continued to proliferate after most of the infected cells died with the release of virions. We examined total DNA from these cells by PCR and found that the culture still contained the infected cells. To estimate the proportion of infected cells, we examined the expression of a viral envelope glycoprotein, gp60/110, by indirect immunofluorescence microscopy, and found that 1% (positive/total, 2/204) of cells expressed the viral protein (Fig. 1A). We also confirmed the percentage of gp60/110-positive cells (1%) by FACS using the same antibody (Fig. 2A). The culture was maintained over a year without significant changes in the ratio of infected (gp60/110 positive) to uninfected (negative) cells (1 to 99).

The ATL cell line, TaY, requires 20 units/ml IL-2 in the culture medium for proliferation. However, it has been shown that IL-2 can inhibit the replication of HHV-6 in mature thymocytes. Therefore, we speculated that a high concentration of IL-2 might suppress HHV-6B replication in the TaY cells. We examined the proportion of infected cells in the culture at 4, 7, 11 and 30 days after reducing the concentration of IL-2 to 5 units/ml. The infected cells were identified by the surface expression of gp60/110. The ratio of infected cells did not change for the initial 4 days, and then increased rapidly between days 7 and 11 (Fig. 2B and 2C). About half of the cells were infected at 11 days after the reduction in the concentration of IL-2. After the burst increase, the ratio of infected cells decreased rapidly before becoming constant at a high level by 30 days after the change. The culture contained 25% infected and 75% uninfected cells and the ratio was maintained thereafter.

Analysis by the indirect immunofluorescence microscopy confirmed the proportion of infected cells in the culture maintained in a lower concentration of IL-2 to be 22% (positive/total, 20/91) (Fig. 1B). When the concentration of IL-2 was reduced to 10 units/ml, the culture showed a similar change with a transient

![A](image1.png)

![B](image2.png)

Fig. 1. Expression of gp60/110 in the HHV-6B-infected ATL cell culture. The expression of the HHV-6B envelope glycoprotein gp60/110 was examined by indirect immunofluorescence microscopy. The proportion of gp60/110-positive cells was 1% (positive/total, 2/204) in the culture maintained in 20 units/ml IL-2 (A) and 20% (20/91) in 5 units/ml IL-2 (B). Representative results are shown. DIC, differential interference contrast microscopy.
increase in the number of infected cells, and another balance of 10% infected and 90% uninfected cells, indicating an IL-2-dependence of the balance in the culture.

To question whether the viral replication was efficient at a lower concentration of IL-2, we compared the amount of viral genome by semiquantitative PCR. Total DNA was prepared from the cell cultures and the 417-bp fragment of the HHV-6B U69 gene was amplified using a series of diluted DNA samples. A similar amount of the DNA fragment was produced using 0.0032 ng of DNA from the cells in 5 units/ml IL-2 and 0.4 ng of DNA in 20 units/ml IL-2 (Fig. 3). We amplified the 262-bp human \( \beta \)-globin gene using these samples and confirmed that the cellular gene was amplified equally from DNA of HHV-6B infected and uninfected cells. Therefore, the amount of viral DNA in the culture increased about 125-fold when the IL-2 concentration was reduced.

HHV-6 infection is sensitive to the antiviral drug ganciclovir, which is phosphorylated by the U69 protein kinase and inhibits viral DNA polymerase\(^{35-37} \). To confirm that the virus was replicating in the culture, we treated the low IL-2 culture (5 units/ml) with 12 \( \mu \)M ganciclovir for 7 days, and examined the proportion of infected cells.

![Fig. 2. IL-2 regulated persistent HHV-6B infection of an ATL cell line.](image)

(A) FACS analysis of gp60/110 expression in the HHV-6B-infected culture (I) and the uninfected parental ATL cell culture (U) maintained in 20 units/ml IL-2. The proportion of gp60/110-positive cells (M1) was 1%. (B) Cells were examined at 0, 4, 7, 11 and 30 days after the IL-2 concentration in the medium was reduced to 5 or 10 units/ml from 20 units/ml. Representative data obtained at 0, 4, 7, and 11 days are shown and indicated by numbers. The proportion of gp60/110-positive cells (M1) were 1%, 1%, 6% and 29% at 0, 4, 7, and 11 days respectively in the culture maintained in 10 units/ml (upper panel), and 1%, 1%, 17% and 61% in the culture of 5 units/ml IL-2 (lower panel). The uninfected parental ATL cell culture was also examined as control (U). (C) Summary of the experiments described in (B). Data represent mean values and standard errors from triplicate experiments.

![Fig. 3. HHV-6B replicated efficiently in the ATL cell culture maintained in a low IL-2 medium.](image)

The HHV-6B U69 gene fragment (417 bp) was amplified from total DNA of the cell cultures by PCR (upper panel). The human \( \beta \)-globin gene fragment (262 bp) was also amplified as control (lower panel). Total DNA was prepared from the infected ATL cell cultures maintained in 5 or 20 units/ml IL-2, and the uninfected parental cells. Amounts of total DNA used for PCR are indicated.
infected cells by FACS. As shown in Figure 4, ganciclovir treatment effectively reduced the number of infected cells in the culture, indicating that the virus replicated efficiently in the culture. As a control experiment, the same culture was treated similarly with 10 μM interferon-γ (IFN-γ) to find an agent that had no effect on the proportion of infected cells.

HHV-6B-infected ATL cells stimulated the growth of the uninfected cells in the culture. The infected cells were stably maintained in the cultures for a long period (1 year), suggesting that they are of some benefit to the uninfected cells. To examine the growth of the cultures, we plated 2 x 10^5 cells in each well of a 24-well plate and enumerated the cells after 3, 7, and 10 days (Fig. 5A). The doubling time of uninfected parental cells was calculated as 45 h in 20 units/ml IL-2. A reduction in the concentration of IL-2 to 5 units/ml resulted in a longer doubling time, 58 h. Interestingly we found that the HHV-6B-infected cultures proliferated faster than the corresponding uninfected cells. The doubling time of the infected culture was 38 h in the presence of 20 units/ml IL-2, and 42 h at 5 units/ml, which corresponds to 16% and 28% acceleration compared to the parental cells at the same IL-2 concentration. To confirm the effect of HHV-6 infection on cell growth, the cells maintained in 20 units/ml IL-2 were washed with PBS and transferred to medium without IL-2. The cell culture containing infected cells continued to grow for 7 days at half the rate of those cultured with IL-2. Most cells were newly infected in 7 days in this culture condition and the extensive release of virion occurred thereafter. Therefore, these cells stopped proliferating and died after 10 days. As a control experiment, we examined the uninfected parental cells similarly and found that they stopped growing immediately (Fig 5B). Thus, HHV-6B-infected cells stimulated the growth of the uninfected cells in the culture.

Discussion

We have demonstrated that HHV-6B establishes a persistent infection in the growing culture of an ATL cell line, TaY. The proportion of infected cells was relatively constant and regulated by the concentration of IL-2 in the culture medium. An analysis of growth rates of the infected cultures revealed that HHV-6B infected cells facilitate efficient growth of the uninfected cells.

Human cytomegalovirus (HCMV), another of the
beta-herpesviruses, propagates efficiently in cells in a very low levels of serum in vitro. It is also reported that the virus infection induces cell cycle arrest. These findings indicate that cell growth suppresses the replication of HCMV and vice versa. The same argument can be made for HHV-6 infection. IL-2 is a potent activator of T cells that stimulates proliferation, and HHV-6 also induces cell cycle arrest. Therefore, it is reasonable that IL-2 inhibits the replication of HHV-6B and regulates a balance between the infected and uninfected cells in a persistent infection of the ATL cell line as demonstrated in this study (Fig. 2).

To our surprise, however, the HHV-6B-infected culture grew faster than the uninfected cells (Fig. 5), because HHV-6 arrests the cell cycle of productively infected cells as mentioned above. Provided that the infected cells do not contribute to the increased number of cells in the culture, the uninfected cells in the culture should be responsible for the net increase in the population. A simple calculation using the doubling time of the culture (Fig. 5) and the proportion of infected cells in the culture (Fig. 2) indicates that the infected cells (25% of all cells) stimulate the uninfected cells (75%) to proliferate at a rate 1.4-fold faster than when the culture was maintained with 5 units/ml IL-2. At 1%, infected cells stimulated the other 99% of cells (uninfected) by 1.2-fold when the culture contained 5 units/ml IL-2. The rapid growth of the uninfected cells was not due to the selection of faster growing cells during long-term culture, because the balance of infected and uninfected cells in the culture can change in a relatively short period (2-3 weeks) by increasing or reducing the concentration of IL-2. Furthermore, we demonstrated that the infected culture can grow for at least 7 days without IL-2. These results suggest that the infected cells secrete factors activating the uninfected cells, or stimulate the uninfected cells via cell-to-cell interactions. Several cytokines are known to stimulate the proliferation of T cells, including IL-2, IL-4, IL-7 and IL-15. An immunomicroarray analysis using the human T-cell lymphoblast line SupT1, however, showed that none of these cytokines are activated by the HHV-6 infection. It is important to examine whether the culture medium conditioned by HHV-6B-infected cells may facilitate growth of uninfected cells.

With the stimulation of growth by the infected cells, the culture neither loses the infected cells nor is overwhelmed by the infection, and succeeds in achieving a balance between the infected and uninfected cells. An analysis of 40 children with acute lymphoblastic or myeloid leukemia indicates a role for HHV-6 infection at the onset of childhood leukemia. HHV-6-infected cells may also facilitate the proliferation of transformed cells nearby in vivo.

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References


