REACTIVATION OF LATENT HUMAN IMMUNODEFICIENCY VIRUS 1 BY HUMAN HERPESVIRUS 6 INFECTION

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Received June 19, 2006; accepted December 15, 2006

Summary. – Infection of the ACH-2 line of human leukemic T cells carrying latent Human immunodeficiency virus 1 (HIV-1) with Human herpesvirus 6 (HHV-6) resulted in an increase in reverse transcriptase (RT) activity, a marker of HIV-1 activation, in the culture supernatant. A similar effect was obtained with 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The RT activity reached a peak at 24 hrs post infection (p.i.) and then declined, suggesting that the cells underwent lysis. The HIV-1 antigen was co-expressed with an early-late HHV-6 product, but not always with an immediate-early (IE) HHV-6 product, suggesting that one or more IE gene products were involved in the activation of latent HIV-1 in ACH-2 cells.

Key words: Human herpesvirus 6; Human immunodeficiency virus 1; reactivation, latent infection

Introduction

HIV-1, the etiological agent of AIDS, causes viremia in infected persons at the beginning of infection, and soon thereafter the virus is cleared from the blood, most likely due to activation of the host cell-mediated immunity (Koup *et al*., 1994). However, the virus remains in the lymphoid tissue in concealment (Embretson *et al*., 1993; Pantaleo *et al*., 1993). The cells of lymphoid tissue bearing the latent virus release a small amount of the virus continuously into the blood for years (Levy, 1993). Years or decades later, the latent virus in these cells is somehow activated and multiplies rapidly to increase its amount in the blood, which leads to the onset of AIDS (Michael *et al*., 1995; Wei *et al*., 1995). Thus, to better understand the pathogenesis of this disease, it is important to clarify the mechanism by which the latent virus is activated.

HHV-6 was first isolated in 1986 from the peripheral blood of patients with lymphoproliferative disorders (Salahuddin *et al*., 1986). There are, genetically and antigenically, two distinct variants of HHV-6, A and B (HHV-6A and HHV-6B) (Ablashi and Salahuddin, 1992). HHV-6A is associated with a lymphoproliferative disease and immunodeficiency including AIDS. HHV-6B was first isolated from an *exanthema subitum* (*roseola infantum*) (Yamanishi *et al*., 1988). It replicates predominantly in CD4+ lymphocytes *in vitro* and *in vivo* (Takahashi *et al*., 1989). Latent HHV-6 resides in peripheral blood leukocytes at various, though relatively low frequencies (Kondo *et al*., 1991; Rajčáni *et al*., 1994). HHV-6B is quickly eliminated from the blood after the acute phase of infection (Kondo *et al*., 1990), but minute quantities of viral DNA remain in a small population of blood leukocytes. Kurata *et al*. (1990) have reported a marked increase in the HHV-6 antigen in the lymphocytes and blood in persistent generalized lymphadenopathy (PGL). The PGL symptoms progress to

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Abbreviations: CAT = chloramphenicol acetyl transferase; CBMCs = cord blood mononuclear cells; CCR = CC-chemokine receptor; CXCR = CXC-chemokine receptor; DR = direct repeat; ES = *exanthema subitum*; gH = glycoprotein H; HHV-6 = Human herpesvirus 6; HIV-1 = Human immunodeficiency virus 1; IE = immediate-early; IFA = immunofluorescence assay; LTR = long terminal repeat; MAb = monoclonal antibody; PGL = persistent generalized lymphadenopathy; PHA = phytohemmaglutinin; p.i. = post infection; RT = reverse transcriptase; TPA = 12-O-tetradecanoyl-phorbol-13-acetate

the manifestation of an AIDS-related complex, at which point, HIV-1 and HHV-6 antigens disappear gradually from atrophic lymphocyte follicles. More recently, the studies using chloramphenicol acetyltransferase (CAT) and luciferase assays have shown that some HHV-6 genes serve as transcriptional activators of long terminal repeats (LTRs) of HIV-1 (Gravel *et al*., 2002, 2003). Thus, it is likely that the HHV-6 infection activates the replication of latent HIV-1. Here, we demonstrate that a superinfection with HHV-6 reactivates latent HIV-1 in HIV-1-bearing human leukemic cells.

Materials and Methods

Cells and virus. Two different cell lines carrying latent HIV-1, U1 from U937 cells (Folks *et al*., 1987) and ACH-2 from CEM cells (Clouse *et al*., 1989), were kindly provided by Dr. T.M. Folks, Centers for Disease Control and Prevention, Atlanta, USA. Another cell line carrying latent HIV-1, MOLT-20-2, which was obtained by infecting MOLT-4 cells with HIV-1 (Fujinaga *et al*., 1995), was kindly provided by Dr. K. Ikuta, Research Institute for Microbial Diseases, Osaka University, Suita, Japan. These cell lines were grown in the RPMI 1640 medium supplemented with 10% of FCS. Umbilical cord blood mononuclear cells (CBMCs) were separated on a Ficoll-Conray gradient and stimulated with 5 μg/ml phytohemagglutinin (PHA) in the growth medium for 2–3 days. The HST strain of HHV-6B, which was isolated from a patient with *exanthema subitum* (ES) (Yamanishi *et al*., 1988; Isegawa *et al*., 1999), was grown in activated CBMCs. To prepare a HHV-6 stock the cultures of PHA-stimulated and HHV-6-infected CBMCs showing CPE in at least 80% of population were frozen-thawed twice and spun at 3,000 rpm for 10 mins. The supernatant was stored at -80°C.

Infection of cells with HHV-6. To carry out the infection 10^6 ACH-2 cells were washed twice with PBS, suspended in 1 ml of the RPMI 1640 medium with 10% FCS containing 10^6 $TCID_{50}$ of HHV-6, and spun in No. 60.542S Assist tubes (Assist, Japan) at 3,000 rpm for 40 mins at 37°C. The cells were then cultured in the growth medium. At different times p.i., the medium was harvested, clarified by centrifugation and stored at -80°C until assayed for RT activity. The harvested cells were subjected to immunofluorescence assay (IFA).

Reactivation of latent HIV-1 with TPA. The cells were cultured in the growth medium with or without 50 ng/ml TPA (Sigma) (Yang *et al*., 1993). At different times of cultivation, the medium was harvested, clarified by centrifugation and stored at -80°C until assayed for RT activity. The harvested cells were subjected to IFA.

IFA. A serum from an HIV-1-seropositive person served as polyclonal antibody to HIV-1 antigen. As the HIV-1 antigen was detectable with this serum diluted even more than 1:10,000 and the HHV-6 antigen could not be detected with an 1:64 dilution of this serum, a 1:1,000 serum dilution was chosen as optimal for testing samples. For HHV-6 gH, a murine monoclonal antibody (MAb) OHV3 diluted 1:3,000 was employed (Okuno *et al*., 1990). For the HHV-6-IE-1 antigen, an 1:250 dilution of hyperimmune serum (anti-IE-1-Abs), prepared by immunizing a SPF rabbit with a purified IE-1 protein (Takeda *et al*., 1996), was used. The cells to be tested by IFA were washed with PBS, spotted on glass slides and fixed with cold acetone. The cells were incubated for 1 hr at room temperature with two combinations of antibodies, (i) the MAb OHV3 plus the anti-HIV-1 serum, and (ii) anti-IE-1-Abs plus the anti-HIV-1 serum, respectively. Afterwards, the cells were washed thoroughly with PBS and incubated for an additional 1 hr with a FITC-conjugated anti-mouse immunoglobulin, an anti-rabbit immunoglobulin diluted 1:60 (Dako, Japan), or a Texas Red-conjugated anti-human immunoglobulin diluted 1:60 (EY Laboratories, USA). Fluorescent images were obtained with a Bio-Rad MRC1024 confocal laser-scanning microscope.

RT assay. The virion-associated RT activity was measured according to Willey *et al*. (1988). Briefly, 10 μl of culture supernatant was mixed with 50 μl of RT reaction mixture consisting of $(A)_n$ (5 μg/ml) and $(dT)_{12\text{-}18}$ (1.57 μg/ml) in 50 mmol/l Tris pH 7.8, 7.5 mmol/l KCl, 2 mmol/l dithiothreitol, 5 mmol/l $MgCl_2$, 0.05% Nonidet P-40, and 0.5 μCi [alpha-^{32}P]dTTP (400 Ci/mmol; Dupont NEN, USA). After incubation at 37°C for 90 mins, 10 μl of the reaction mixture was spotted onto DEAE ion-exchange paper (Whatman, USA) and washed 5 times with 2x SSC to remove unincorporated label. The spots were visualized and quantified using an imaging analyzer BAS-3000 (Fujix, Japan).

Results

HIV-1-associated RT activity in the supernatant of cultures of various cell lines after stimulation with HHV-6 and TPA

Three cell lines carrying latent HIV-1, namely ACH-2, U1, and MOLT-20-2 were treated with TPA to prove the presence of latent HIV-1 and the absence of latent HHV-6. The treatment induced RT activity, a marker of HIV-1, in the culture supernatants of all these cell lines (Fig. 1). The activity reached a maximum at the day 5 of the treatment and then declined. On the other hand, the HHV-6 antigen was not detected.

As regards the stimulation of the cells with HHV-6, the infection resulted in the positivity of 5–15% of cells for the HHV-6 antigen by IFA, while U1 and MOLT-20-2 cells did not show any significant increase in the positivity (<1%). Therefore, in further experiments, ACH-2 cells were employed. The infection of ACH-2 cells with HHV-6 caused an increase in RT activity in the culture supernatant, but the time course was different from that caused by TPA. The RT activity in HHV-6-infected cells increased first at 12 hrs, peaked at 24 hrs p.i., and then declined. On the other hand, the RT activity in TPA-treated cells increased first at 24 hrs, peaked at day 5 of the treatment and then declined.

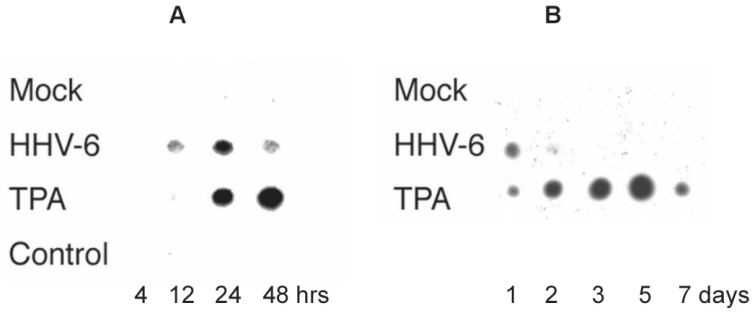

Fig. 1

Detection of HIV-1 associated RT activity in the supernatant of cultures of ACH-2 cells after stimulation with HHV-6 and TPA

RT activity was assayed at days 4–48 hrs (A) and days 1–7 (B) post stimulation with HHV-6 and TPA, respectively. Non-treated (control) and mock-infected cells represented controls.

Presence of HIV-1 and HHV-6 antigens in ACH-2 cells after stimulation with HHV-6 and TPA

The time course of increase in RT activity in the culture supernatant of ACH-2 cells coincided with that of the presence of the HIV-1 antigen. The latter appeared in the cells first at 12 hrs and disappeared at 72 hrs p.i. (Fig. 2). Moreover, the HIV-1-antigen-positive cells co-expressed an early-late HHV-6 gH antigen (Fig. 3). These results indicated that the HHV-6 infection activated latent HIV-1 to liberate virion-associated RT into the culture supernatant.

Relationship between HIV-1 and HHV-6 antigens in ACH-2 cells after HHV-6 infection

The expression of the immediate-early IE-1 and early-late gH antigens of HHV-6 in relation to that of the HIV-1 antigen in ACH-2 cells was followed (Fig. 4). The expression of the HHV-6 IE-1 antigen was not strictly associated with that of the HIV-1 antigen, while the expression of the early-late HHV-6 gH antigen was always associated with that of the HIV-1 antigen. The existence of IE-1-positive cells lacking the HIV-1 antigen indicated that the expression of immediate-early HHV-6 genes preceded the activation of latent HIV-1, suggesting that certain immediate-early gene products of HHV-6 play an important role in the activation of latent HIV-1 in ACH-2 cells.

Discussion

Although HHV-6 and HIV-1 are different viruses, they have some similar characteristics, e.g. the T-cell tropism. There are many reports about interactions between HHV-6 and HIV-1, in particular about the role of HHV-6 in the activation of HIV-1 replication. Some of them regard HHV-6 as an activator (Ensoli *et al*., 1989; Lusso *et al*., 1989, 1991, 1995; Vignoli *et al*., 2000; Csoma *et al*., 2002), while others

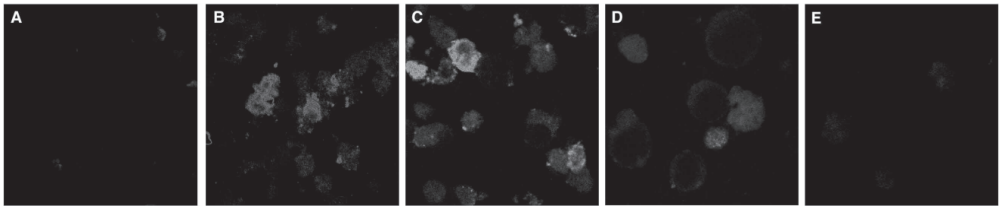

Fig. 2

Detection of HIV-1 antigen in HHV-6-infected ACH-2 cells

IFA carried out at 4, 12, 24, 48, and 72 hrs p.i. with HHV-6 (A–E).

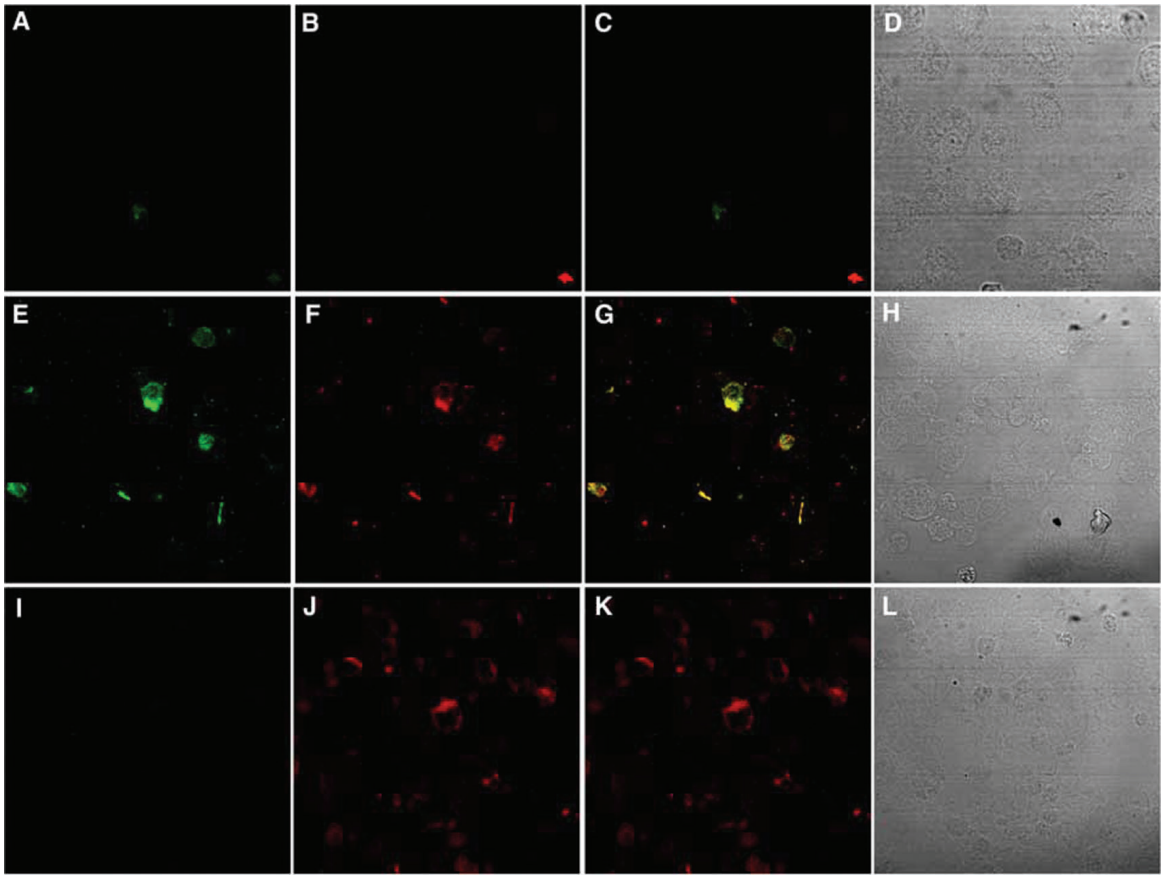

Fig. 3

Detection of HIV-1 and HHV-6 antigens in ACH-2 cells stimulated with HHV-6 and TPA

IFA was carried out at 24 hrs post stimulation with HHV-6 and TPA, respectively. Green and red fluorescence correspond to HHV-6 and HIV-1 antigens, respectively. Mock-infected cells (A–D), HHV-6 infected cells (E–H), and TPA-stimulated cells (I–L). HHV-6 antigen was detected with the MAb OHV-3 (A, E, and I) and the HIV-1 antigen was detected with the HIV-1 antiserum (B, F, and J). Merged images of A and B (C), E and F (G), and I and J (K), respectively. Light microscopy of the same cells (D, H, and L).

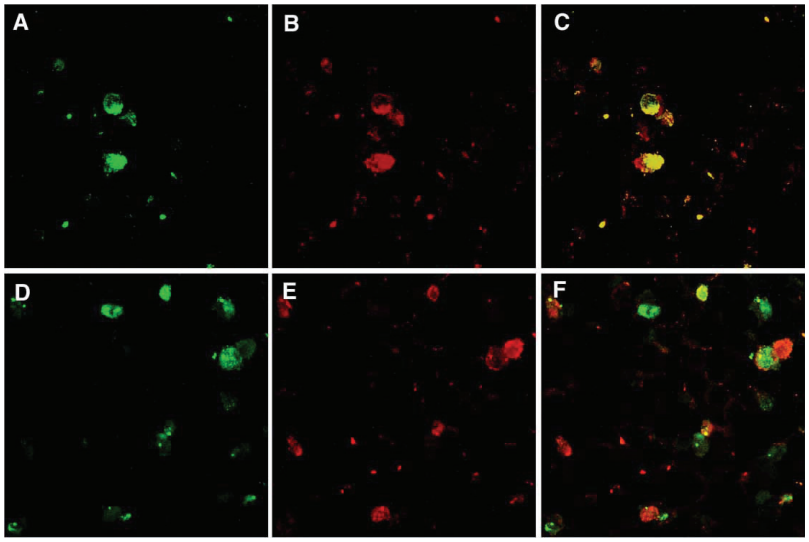

Fig. 4

Detection of HHV-6 gH, HHV-6 IE-1, and HIV-1 antigens in HHV-6-infected ACH-2 cells

IFA was carried out at 24 hrs p.i. with HHV-6. Green and red fluorescence correspond to HHV-6 and HIBV-1 antigens, respectively. HHV-6-gH antigen was detected with the MAb OHV-3 (A) and the HIV-1 antigen was detected with HIV-1 antiserum (B and E). The HHV-6 IE-1 antigen was detected with the rabbit serum (D). Merged images of A and B (C) and D and E (F), respectively.

as a suppressor of this phenomenon (Carrigan *et al*., 1990; Grivel *et al*., 2001) We showed that latent HIV-1 in ACH-2 cells was activated by superinfection with HHV-6.

Poli and Fauci (1992) have reported that cytokines are important for HIV-1 replication in lymphocytes and mononuclear phagocytes. However, we do not assume that cytokines played an essential role in the activation of latent HIV-1 in our experimental system, because we did not observe any HIV-1 antigen-positive and at the same time HHV-6 antigen-negative cells. Furthermore, Gobbi *et al*. (2000), in studying SCID-hu Thy/Liv mice co-infected with HHV-6 and HIV-1, expected that direct interactions between the two viruses might alter their replication or pathogenicity; however, they found that the HIV-1 load was either unchanged or reduced in co-infected implants compared with those infected with HIV-1 alone. The overall lack of dramatic effects induced by the co-infection with HHV-6 and HIV-1 in SCID-hu Thy/Liv mice suggested that the two viruses replicated simultaneously without affecting each other, possibly indicating that they infected different cells. The results of our study indicate that one or more intracellular events following the infection with HHV-6 are essential for the activation of latent HIV-1.

Ensoli *et al*. (1989) have reported that a co-infection of CD4+ lymphocytes with HIV-1 and HHV-6 accelerates cell death. Thus, it is likely that the decrease in RT activity in the culture supernatant and the disappearance of cells positive for both HIV-1 and HHV-6 antigens observed in our study were caused by the lysis of co-infected cells. It is not surprising that we did not observe any IE-1-negative cells expressing the HIV-1 antigen, because the IE-1 antigen is expressed for only a limited period of infection (Isegawa *et al*., 1998).

Although HIV-1 activation from latency has been suggested to be linked to HIV-1 superinfection (Kishi *et al*., 1995; Robertson *et al*., 1995), in the case of HHV-6-superinfected ACH-2 cells investigated in our study, HIV-1 could be reactivated by component(s) expressed by HHV-6.

In T lymphocytes, a co-infection of CD4+ cells with HHV-6 and HIV-1 accelerated HIV-1 expression and led to cell death (Lusso *et al*., 1989; Ensoli *et al*., 1989). In addition, HHV-6 induced a *de novo* expression of CD4 protein in normal mature CD8+ cells, rendering them susceptible to infection with HIV-1 (Lusso *et al*., 1991, 1995). Vignoli *et al*. (2000) have reported that human hematopoietic progenitor cell (HPC) lines infected with HHV-6 show susceptibility to HIV-1 infection, and have suggested two different mechanisms for this phenomenon. HHV-6 infection may increase the susceptibility to HIV-1 infection either by induction of consistent levels of CD4 molecules or by topological rearrangement of cell-surface molecules. The reactivation of latent HIV-1 by superinfection with HHV-6 may differ from the two mechanisms and be caused by a direct induction of gene expression. Carrigan *et al*. (1990) have suggested that HHV-6 may slow the progression of the disease in some HIV-1-infected individuals. In support of this idea, HHV-6 was found to block the infection of macrophages with CCR5-tropic HIV-1 (Grivel *et al*., 2001). The target cell type seems to be important for the outcome of the HIV-1/HHV-6 interaction (Asada *et al*., 1999). These findings suggest that HHV-6 might activate and/or support the growth of CXCR4-tropic HIV-1 in its target cells but might inhibit and/or block the growth of CCR5-tropic HIV-1 in its target cells. In CD4+ cells, the reactivation of latent HIV-1 could be initiated by superinfection with HHV-6.

Based on the results of *in vitro* LTR-CAT or LTR-luciferase assays, at least eight HHV-6 genes that might serve as transcriptional activators have been defined: DR7, U3, U16/U17, U18/U19, U27, U86, U89, and U94 (Geng *et al*., 1992; Martin *et al*., 1991; Mori *et al*., 1998; Nicholas and Martin, 1994; Thompson *et al*., 1994; Thomson *et al*., 1994; Zhou *et al*., 1994). The U16/U17, U18/U19, U86, and U89 gene products are reported to induce HIV-1 replication (Csoma *et al*., 2002).

In this report, we show for the first time that the HHV-6 superinfection of ACH-2 cells reactivates latent HIV-1 and that this reactivation occurs only in HHV-6-infected cells. Because HIV-1 proteins and early HHV-6 proteins were simultaneously expressed in HHV-6-infected ACH-2 cells, HHV-6 IE protein(s) could initiate the reactivation of HIV-1. There are reports on the activation of HIV-LTR with the HHV-6 IE-1 protein (Gravel *et al*., 2002) and the HCMV IE-2 protein, a homolog of the HHV-6-IE-2 protein (Walker *et al*., 1992). These results suggest that, in case of HHV-6 superinfection, the HHV-6 U86 and/or U89 gene products could reactivate latent HIV-1 via HIV-LTR.

Csoma *et al*. (2002) have reported that a co-infection of syncytiotrophoblast cells with HHV-6A and HIV-1 results in the production of infectious HIV-1, while only early gene products appear in the cells infected with HHV-6A alone, and no viral proteins are formed in the cells infected with HIV-1 alone. The authors have suggested that the reactivation of latent HIV-1 in the co-infected cells is mediated by the IE-A and IE-B gene products of HHV-6A, and that a co-infection of syncytiotrophoblast cells with HHV-6A and HIV-1 *in vivo* could contribute to the trans-placental transmission of HIV-1, but not to that of HHV-6A.

Our results presented here are consistent with those of Gravel *et al*. (2002, 2003) and Csoma *et al*. (2002), suggesting that the infection with HHV-6 may induce the replication of latent HIV-1. Our results also suggest that IE gene product(s) of HHV-6 are involved in the activation of latent HIV-1 in the cells. The issue of concrete gene products is presently under study.

We observed the highest HHV-6 gH-expression at day 1 p.i. with HHV-6 and its decline at day 3; neither HHV-6 gH nor HIV-1 RT activity were detected at day 3 p.i. Takasaki *et al*. (1997) have reported a more active HIV-1 replication in HHV-6-superinfected cells compared with the cells infected with HIV-1 alone. They have claimed that HIV-1 might activate cytolytic HHV-6 infection, consequently suppressing the assembly of HIV-1 by cytolysis. These results suggest that the HHV-6 superinfection performed in our study by the centrifugation method resulted in just one growth cycle of HHV-6, which ended without producing infectious virus progeny (Csoma *et al*., 2002).

We observed that the time course of HIV-1 RT activity in the supernatant of ACH-2 cell cultures was similar to that of HHV-6 gH expression. This suggests that the reactivation of HIV-1 was not initiated by cell-free mature HIV-1, but by HHV-6. It is possible that the infected cells did not survive or HHV-6 became latent after one growth cycle. Given that a co-infection of CD4+ lymphocytes with HIV-1 and HHV-6 leads to an accelerated HIV-1 expression and cell death (Lusso *et al*., 1989; Ensoli *et al*., 1989), HHV-6-infected ACH-2 cells could die after the reactivation of HIV-1, or free HHV-6 virions produced in ACH-2 cells could be incapable of secondary infection of other cells.

Kurata *et al*. (1990) have reported the presence of the HHV-6 antigen clearly associated with the activation of HIV-1 at early stage of clinical onset of PGL; however, antigens of other herpesviruses were not detected. The authors suggested that the activation of HIV-1 infection several years after cell entry simultaneously activated latent HHV-6 by immunosuppression or vice versa, and subsequently, the reactivated HHV-6 enhanced the replication of HIV-1. There is also an electron microscopic evidence on the replication of HHV-6 and HIV-1 in the same cells *in vitro* (S. Nii, personal communication). Further studies exploring the interaction between HIV-1 and HHV-6 *in vivo* may provide new insights into the molecular mechanisms underlying the development of AIDS and suggest new therapeutic approaches to block the progression of the disease.

Acknowledgements. This study was supported in part by the Ministry of Education, Welfare, and Culture of Japan.

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