ELECTRONMICROSCOPIC STUDY OF HUMAN HERPESVIRUS 6-INFECTED HUMAN T CELL LINES SUPERINFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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Summary. – Human herpesvirus 6 (HHV-6) has been proposed as one of the co-factors responsible for the development of acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus (HIV) carriers. We analyzed the interaction between HHV-6 and HIV-1 in superinfected cells. Cell-free HIV-1 could superinfect human T cell lines, MT-4 and Molt-4, which had been previously infected with HHV-6. Both HIV-1 and HHV-6 replicated in the same cells. We observed two types of morphologically distinguished cells as early as 4 days after superinfection. One type (D) was degenerate cells with intracellular and extracellular HHV-6 and with less HIV-1 virions. The other type (I) was relatively intact cells with both HIV-1 and HHV-6 virions. Replication of HIV-1 was more active in the type I as compared with type D cells. The level of HIV-1 reverse transcriptase (RT) activity in the culture supernatants of cells superinfected on day 0 declined after day 7, while that in the supernatants of cell cultures infected with HIV-1 alone remained high between days 12 and 40. These results suggest that the superinfection of the HHV-6-infected cells with HIV-1 may induce a degenerative process in these cells.

Key words: human herpesvirus 6; human immunodeficiency virus type 1; superinfection; human T cell line; electron microscopy; reverse transcriptase

Introduction

HHV-6 has been first isolated from patients with lymphocytic disorders (Salauddin *et al.*, 1986) and identified as the etiological agent of *exanthem subitum* (Yamanishi *et al.*, 1988). On the basis of the observation that HHV-6 is frequently isolated from HIV carriers (Agute *et al.*, 1988; Lopez *et al.*, 1988; Salauddin *et al.*, 1986; Tedder *et al.*, 1987) it has been proposed that HHV-6 may be one of the co-factors responsible for the development of AIDS.

Human CD4-positive cells are susceptible to infection with HIV-1 and HHV-6 (Dalgleish *et al.*, 1984; Klazmann *et al.*, 1984; Lusso *et al.*, 1988). There are papers which describe the co-infection/superinfection of cells with HHV-6 and HIV-1 (Carrigan *et al.*, 1990, Ensoli *et al.*, 1989; Horvat *et al.*, 1989; Levy *et al.*, 1990; Lusso *et al.*, 1989). Lusso *et al.* (1989) have reported the presence of both viruses in peripheral blood mononuclear cells (PBMC) 8 days after their simultaneous infection with HIV-1 and HHV-6. Nii *et al.* (1990) showed morphologically that HIV-1 and HHV-6 coexisted in single cells after a co-cultivation of HIV-1-infected cells and HHV-6-infected cells. However, it was not shown whether the cells bearing both HIV-1 and

Abbreviations: AIDS = acquired immunodeficiency syndrome; CAT = chloramphenicol acetyltransferase; EM = electron microscopy; FBS = foetal bovine serum; FITC = fluorescein isothiocyanate; HHV-6 = human herpesvirus 6; HIV = human immunodeficiency virus; IGEM = immunocolloidal gold EM; PBS = phosphate-buffered saline; p.i. = post infection; RITC = tetramethyl rhodamine isothiocyanate; RT = reverse transcriptase

HHV-6 were generated by cell fusion between HIV-1-infected and HHV-6-infected cells or by infection with cell-free viruses.

It is assumed that most of the HIV-1-infected adults had been exposed and sub-clinically infected with HHV-6 before HIV-1-infection, because HHV-6 usually infects individuals early in life and is highly prevalent in adult populations (Fox *et al.*, 1990; Kondo *et al.*, 1991; Okuno *et al.*, 1989; Okuno *et al.*, 1990). It is not known whether HIV-1 reactivates a latent HHV-6 infection and the reactivation of HHV-6 by HIV-1 has any role in the development of clinical symptoms of AIDS, such as unknown skin rash.

In the present study, using electron microscopy (EM), we examined the production of HIV-1 and HHV-6 virions in the cells which were first infected with HHV-6 and then superinfected with HIV-1, and elucidated the interaction between HHV-6 and HIV-1 at the single cell level.

Materials and Methods

Viruses and cells. HHV-6 Hashimoto strain (kindly provided by Prof. K.Yamanishi, Osaka University) was inoculated to MT-4 and Molt-4 cells, which were then cultured with RPMI-1640 medium containing 5% heat-inactivated foetal bovine serum (FBS). Uninfected cells were cultured at the same conditions as control. 1024 $TCID_{50}$ of HIV-1 LAV-1 strain in 100 µl was inoculated to 5 x 10^6 HHV-6-infected cells on day 7 after inoculation of HHV-6. The day of infection with HIV-1 is regarded as day 0. Both viruses were filtered through a Millipore filter unit (pore diameter 0.22 µm) before the inoculation. The superinfected cells, HIV-1-infected cells, HHV-6-infected cells and uninfected cells were then cultured in RPMI-1640 medium containing 10% FBS. A hundred cells were examined in each sample.

We use the term of superinfection throughout this article, because the inoculation of the first virus preceded that of the second one by a definite time period. The term of co-infection is reserved for a case of simultaneous inoculation of two different viruses.

Electron microscopy. The cells were fixed in 2% glutaraldehyde in 0.1 mol/l HCl-sodium cacodylate buffer pH 7.2 at 4°C, and then treated with 1% osmium tetraoxide in the same buffer at 4°C for 2 hrs. The samples were dehydrated through an ethanol series, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate, and examined in a Hitachi H-7100 transmission electron microscope at 100 kV.

Immunocolloidal gold electron microscopy (IGEM). Ultrathin sections were collected on nickel grids and processed for immunochemical labelling. Briefly, they were incubated with a drop of mouse anti-HIV-1 (HTLV-III) gp120 monoclonal antibody (MoAb) (Chemicon Int. Inc., CA, USA) at room temperature for 1 hr, washed in phosphate-buffered saline (PBS) and incubated with a drop of anti-mouse goat IgG conjugated with gold (IgG-gold 5 nm, Amersham) for 60 mins. The sections were then washed in PBS followed by distilled water, stained with uranyl acetate and lead citrate, and observed under the electron microscope.

Immunofluorescence studies. Coverslip cell cultures were fixed in acetone at -20°C for 10 mins and stained by an indirect immunofluorescence method. The mouse anti-HIV-1 gp120 MoAb was added to the fixed cells and the cells were incubated at 37°C for 30 mins. After double washing in PBS, tetramethyl rhodamine isothiocyanate (RITC)-labelled goat anti-mouse IgG was added to the cells and incubation was carried out at 37°C for 30 mins. For the detection of HHV-6 antigens, a HHV-6 MoAb (OHV-1, kindly provided by prof. K. Yamanishi, Osaka University) was added to the cells and the incubation was performed at 37°C for 30 mins. After double washing in PBS, a fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG was added to the cells and the incubation was carried out at 37°C for 30 mins. Two hundred cells were checked, and the percentage of viral antigen-positive cells was calculated.

Reverse transcriptase (RT) assay. HIV-1 RT activity in culture supernatants was tested by RT assay. Culture supernatants of HIV-1-infected MT-4 and Molt-4 cells were used as positive controls. Culture supernatants of MT-4 cells and Molt-4 cells which were infected with HHV-6 alone were used as negative controls. The RT assay was performed according to the procedure reported previously (Lee *et al.*, 1987). Ten µCi of [^{3}H]thymidine triphosphate (64 µCi/mmole, ICN) and 5 µU of poly(rA).oligo(dT) template primer were used.

Results

Presence of HIV-1 and HHV-6 particles in single cells after superinfection

MT-4 cells were infected with HHV-6 and cultivated for 7 days. Eighty-five % of these cells were positive for HHV-6 antigen as determined by the immunofluorescence method. The HHV-6-infected MT-4 cells were then superinfected with HIV-1. On day 4 after superinfection, HIV-1 particles budding from cells were observed by EM. HIV-1 virions were produced by budding with crescent structures on the surface of the cells which also contained many HHV-6 particles in the cytoplasm and nucleoplasm (Fig. 1). The budding particles were determined to be HIV-1 by IGEM with the anti-HIV-1 gp120 MoAb (Fig. 2). We did not find multinuclear cells bearing both HIV-1 and HHV-6 virions in these studies.

Presence of two types of morphologically distinguished cells in superinfected cell cultures

On days 4, 7 and 10 after the superinfection, we observed two types of morphologically distinguished cells (Figs. 3,4). Of type D were degenerated cells which possessed many intracellular and extracellular HHV-6 particles but a lesser

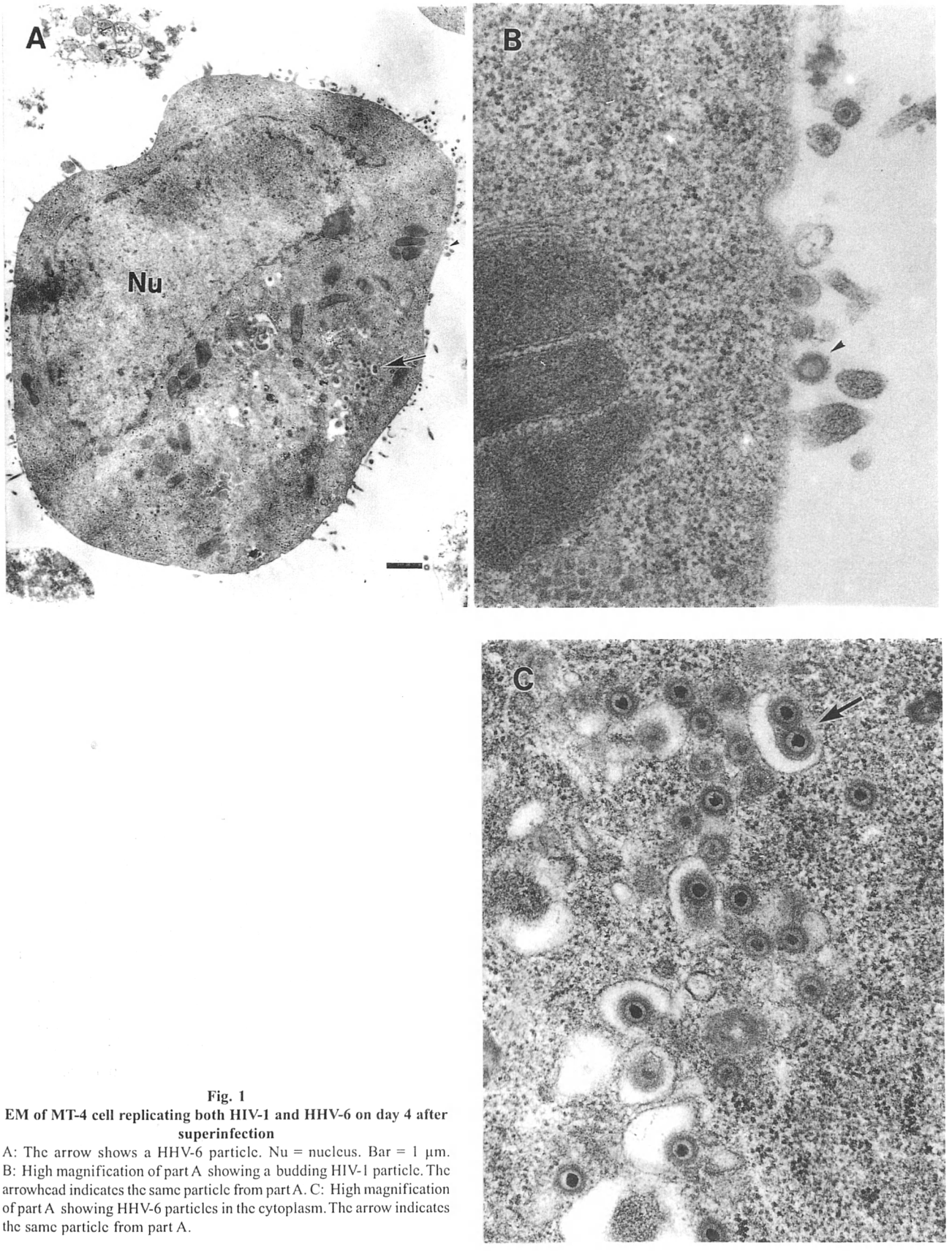

Fig. 1
EM of MT-4 cell replicating both HIV-1 and HHV-6 on day 4 after superinfection

A: The arrow shows a HHV-6 particle. Nu = nucleus. Bar = 1 μm. B: High magnification of part A showing a budding HIV-1 particle. The arrowhead indicates the same particle from part A. C: High magnification of part A showing HHV-6 particles in the cytoplasm. The arrow indicates the same particle from part A.

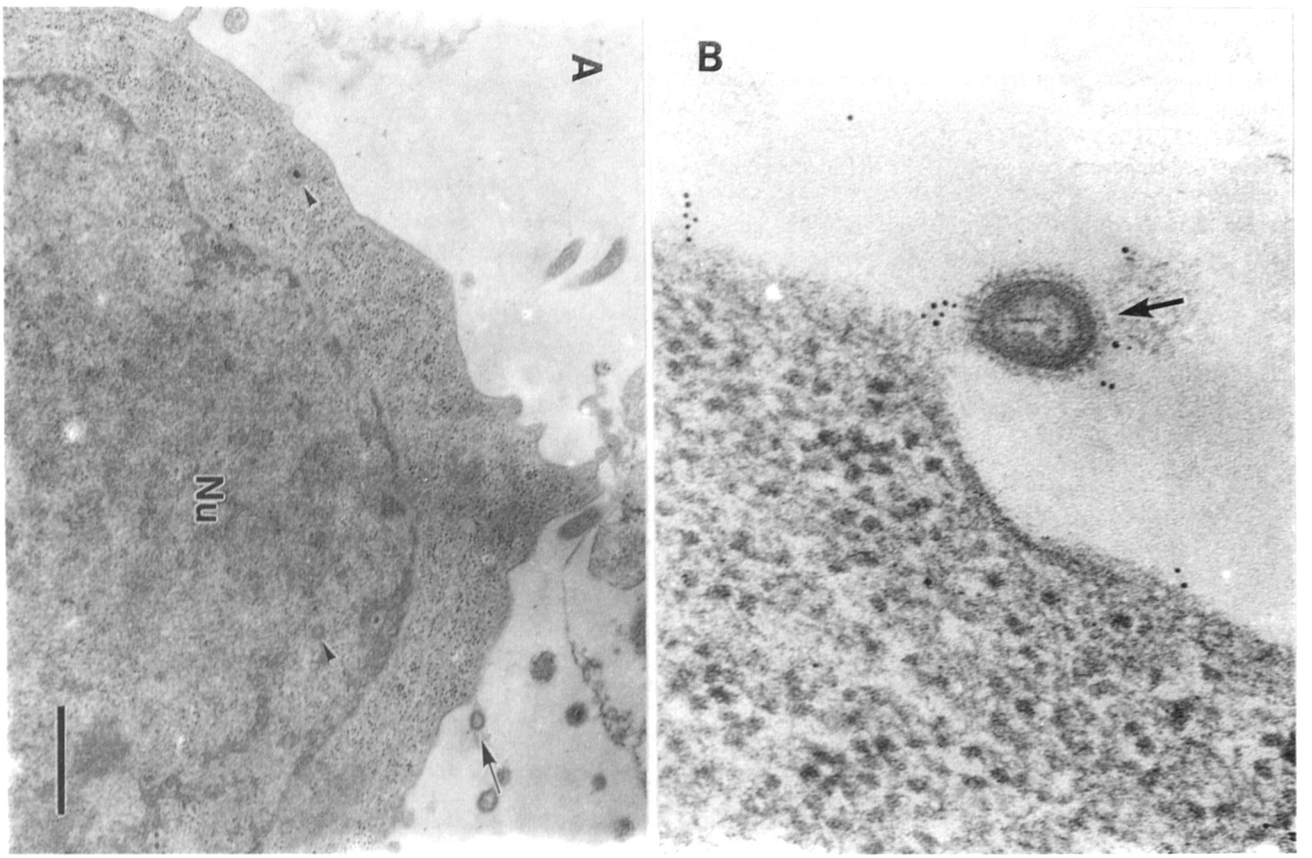

Fig. 2
Confirmation of HIV-1 nature of a budding virus particle by IGEM
Day 4 after superinfection of MT-4 cells with HIV-1. A: The arrowheads indicate HHV-6 particles. Nu = nucleus. Bar = 1 μm. B: The arrow indicates a budding HIV-1 particle.

number of HIV-1 particles (Fig. 3). Of type I were the cells with relatively intact morphology and HIV-1 virions on the cell surface (Fig. 4). Most of type I cells had some vacuoles in the cytoplasm. HHV-6 and HIV-1 virions were found to coexist in some type I cells; however, the number of HHV-6 particles in the type I cells, which were actively replicating HIV-1, was smaller as compared with that of type D cells. The percentage of type D cells in the superinfected culture was slightly higher than that in the cell cultures infected either with HHV-6 or HIV-1 alone (Fig. 4, Table 1).

Levels of RT in the culture supernatants of superinfected cells

We then examined the levels of RT activity in culture supernatants of MT-4 cells infected with both HIV-1 and HHV-6 or HHV-6 alone or HIV-1 alone. The RT activity in the culture supernatants of MT-4 cells infected with HIV-1 alone was first detected on day 7, and was kept at high levels between days 12 and 40 (Fig. 6). The RT activity in the culture fluids of superinfected MT-4 cells increased until day 7; however, it declined gradually and remained at very low levels after day 18. The patterns of the levels of RT activity was similar in infected Molt-4 cells (Fig. 7).

On the other hand, the percentage of HIV-1-positive cells in superinfected MT-4 cells was 72% on day 10 according to the immunofluorescence method, while the percentage of HIV-1-positive cells in MT-4 cells infected with HIV-1 alone was 60%. These results suggest that MT-4 cells infected with both HIV-1 and HHV-6 may degenerate rapidly and the number of HIV-1-positive cells may decline.

Discussion

Human CD4-positive cells are susceptible to an infection with HIV-1 and HHV-6 (Dalgleish *et al.*, 1984; Klazmann *et al.*, 1984; Lusso *et al.*, 1988). MT-4 cells, which

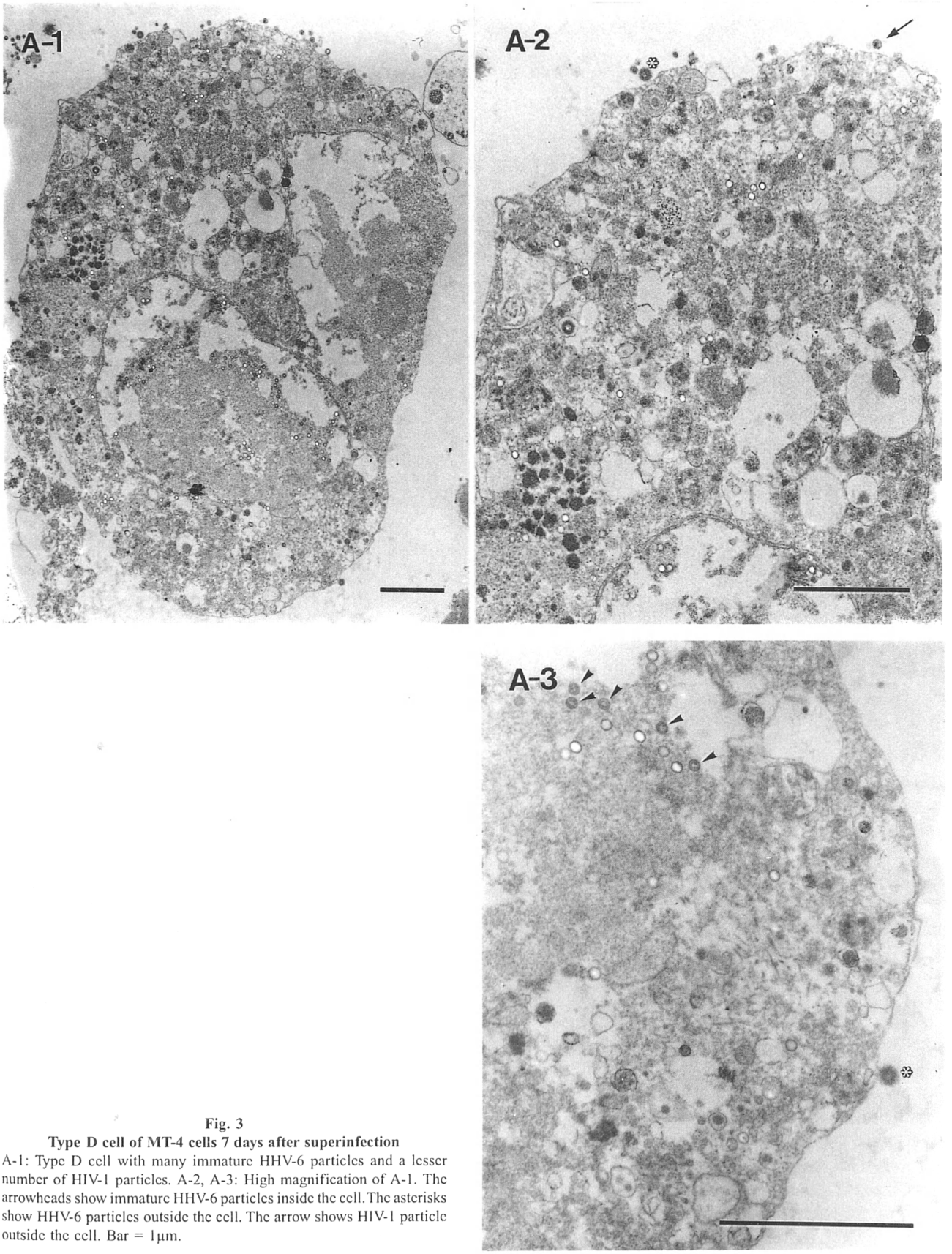

Fig. 3
Type D cell of MT-4 cells 7 days after superinfection
A-1: Type D cell with many immature HHV-6 particles and a lesser number of HIV-1 particles. A-2, A-3: High magnification of A-1. The arrowheads show immature HHV-6 particles inside the cell. The asterisks show HHV-6 particles outside the cell. The arrow shows HIV-1 particle outside the cell. Bar = 1μm.

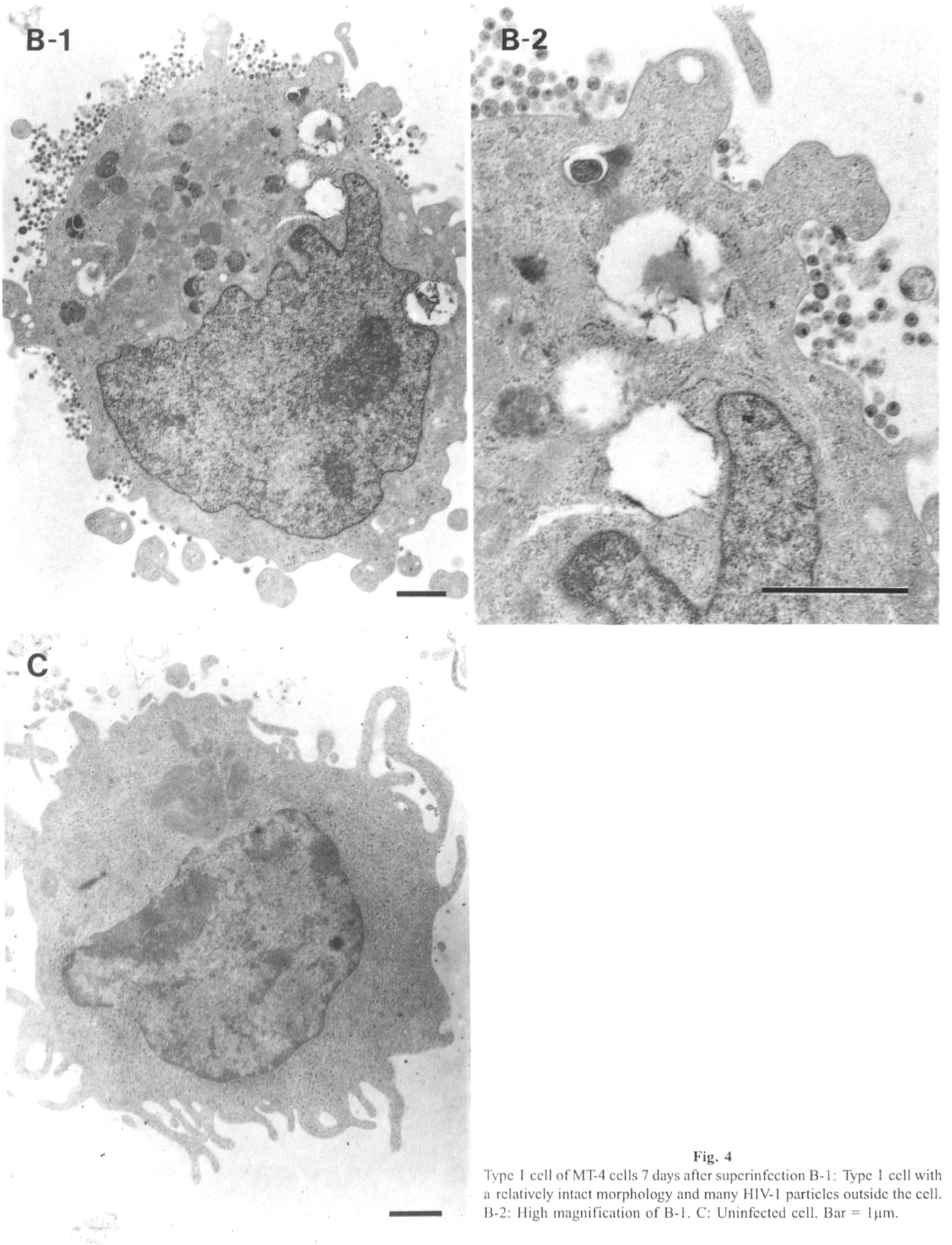

Fig. 4
Type I cell of MT-4 cells 7 days after superinfection B-1: Type I cell with a relatively intact morphology and many HIV-1 particles outside the cell. B-2: High magnification of B-1. C: Uninfected cell. Bar = 1μm.

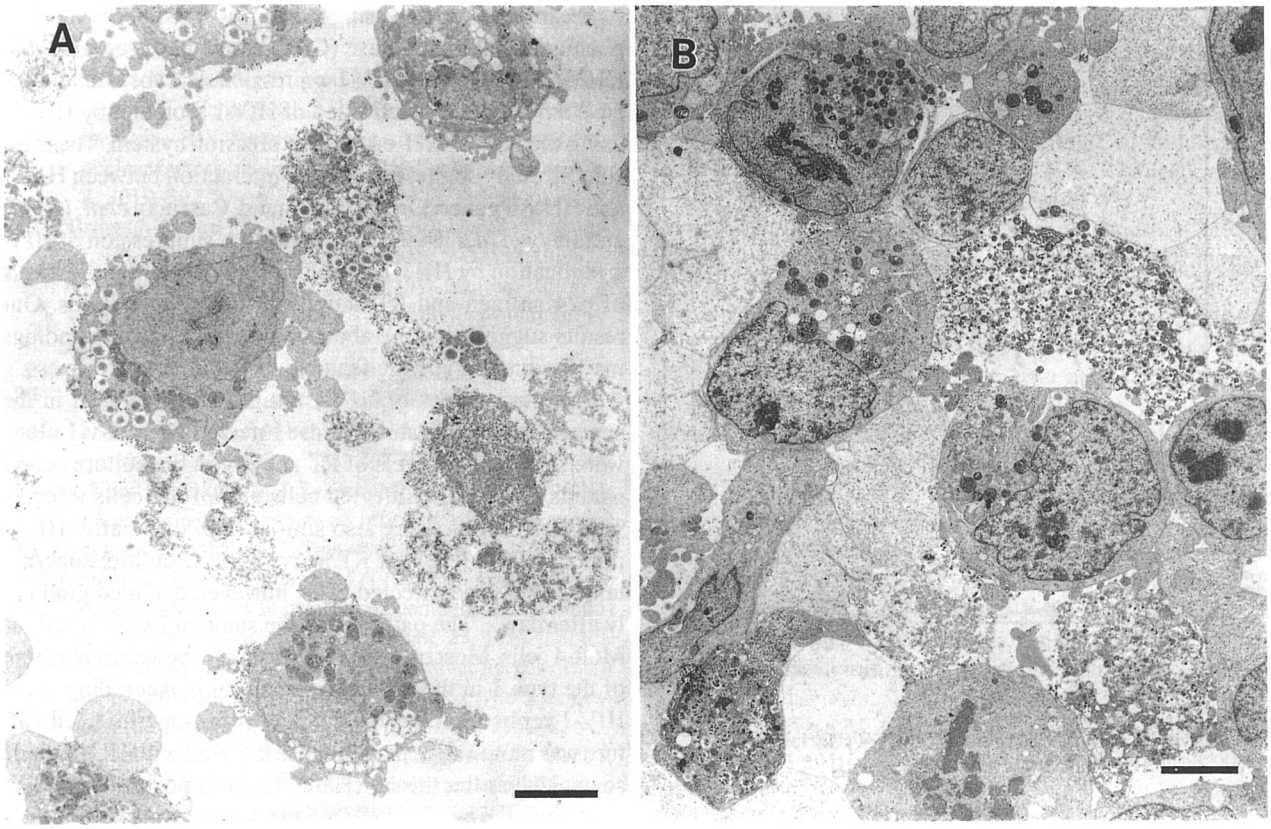

Fig. 5
Frequency of the occurrence of the type D cells in MT-4 cells infected with HHV-6 alone and with HHV-6 plus HIV-1 A: HHV-6-infected cells superinfected with HIV-1. Day 7 after superinfection. A frequent occurrence of the type D cells. B: Cells infected with HHV-6 alone. Day 14 after infection. Bar = 5 μm.

are an HTLV-I-infected cell line, were reported to be the most susceptible to HHV-6 infection among seven T cell lines examined: MT-4, HPB-ALL, Molt-3, Molt-4, CCRF-CEM, HUT-78 and H-7 (Asada *et al.*, 1989). Therefore, we chose MT-4 cells to analyze the interaction between HHV-6 and HIV-1. In some experiments we used also another human T cell line, Molt-4.

The co-existence of HIV-1 and HHV-6 in the same cells was demonstrated after co-cultivation of HIV-1-infected human T cell lines and HHV-6-infected human cord blood lymphocytes (Nii *et al.*, 1990). It has also been shown that HIV-1 and HHV-6 coexisted in single activated lymphocytes on the 8th day of culture after simultaneous inoculation of both viruses (Lusso *et al.*,1989). However, it has not been confirmed that the cells bearing HHV-6 particles could be infected with cell-free HIV-1 or that the cells bearing both HIV-1 and HHV-6 particles may be generated by the fusion of HIV-1-infected and HHV-6-infected cells. In the present study, we demonstrated that (1) both HIV-1 and HHV-6 virions were assembled in cells with a single nucleus 4 days after superinfection, and (2) HIV-1 particles were present on the surface of the cells which contained also HHV-6 particles in the cytoplasm and nucleoplasm. These findings indicate that cell-free HIV-1 can superinfect HHV-6-bearing cells and replicate.

Table 1. Percentage of the D type cells in MT-4 cell cultures infected with HHV-6 or HIV-1 or HHV-6 plus HIV-1 assayed by EM

Days in culture*	Percentage of the D type cells infected with			
	HHV-6 + HIV-1	HHV-6	HIV-1	– (uninfected culture)
0	32(22)	10		
4	74(65)	70(61)	12(3)	9
7	60(52)	40(32)	17(9)	8
10	54(47)	30(22)	14(6)	8

A hundred cells were observed in each sample. The numbers in parentheses show percentages after subtracting the percentage of degenerated cells in uninfected culture. *After HIV infection.

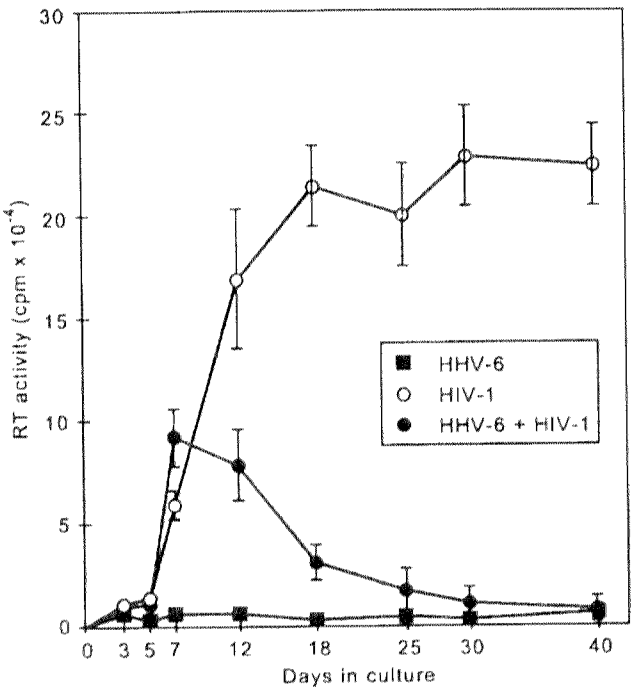

Fig. 6
RT activity in culture supernatants of MT-4 cells infected with HHV-6 or HIV-1 or HHV-6 plus HIV-1

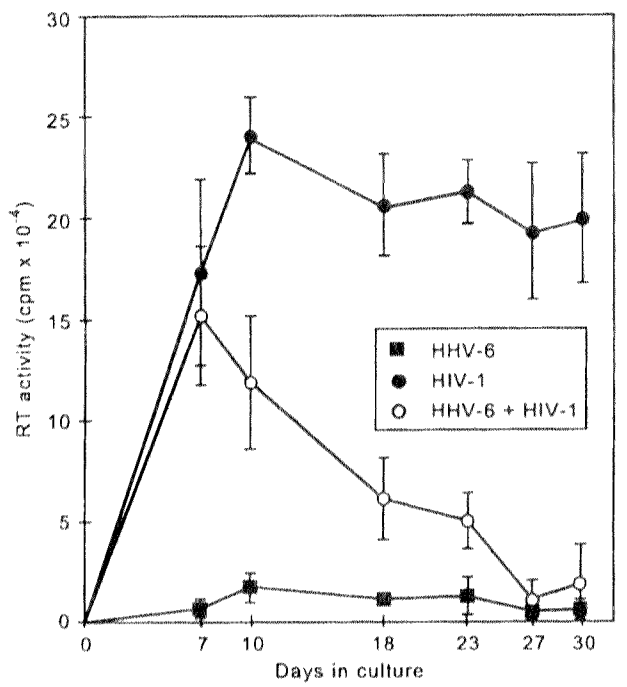

Fig. 7
RT activity in culture supernatants of Molt-4 cells infected with HHV-6 or HIV-1 or HHV-6 plus HIV-1

Ensoli *et al.* (1989), using the HIV-1 LTR-linked chloramphenicol acetyltransferase (CAT) assay, have reported that HHV-6 increased the HIV-1 expression. Harvot *et al.* (1989) have reported a transactivation of HIV-1 promoter by HHV-6 using the HIV-1 CAT enzyme expression system. These results strongly suggest a positive interaction between HIV-1 and HHV-6 genes. On the other hand, Carrigan *et al.* (1990) and Levy *et al.* (1990) have reported a suppression of HIV-1 replication by HHV-6 in cultures by measuring the levels of p24 antigen and RT activity in the culture fluids. Our results suggest that the abovementioned opposite findings may be due to dynamic features of the infection process.

The percentages of HIV-1 antigen-positive cells in the superinfected cells and in those infected with HIV-1 alone were similar. The levels of RT activity in the culture supernatants of the superinfected cells and of the cells infected with HIV-1 alone were also similar until day 7 after HIV-1 infection. The levels of RT activity in the culture supernatants of the superinfected cells, however, declined gradually after day 7. The pattern was the same in MT-4 as well as Molt-4 cells. Most of the HIV-1 particle-producing cells were of the type I in the superinfected culture. Accordingly, the HIV-1 replication in type I cells of the superinfected culture was more active than in those infected with HIV-1 alone several days after the superinfection. It is possible that HIV-1 activates the cytolytic HHV-6 production and consequently the assembly of HIV-1 is suppressed by the cytolysis. The present study suggests the possibility that HHV-6 may play a role of a co-factor in HIV-1 infection. Further study is necessary to elucidate the interaction between HIV-1 and HHV-6, and the role of HHV-6 in the pathogenesis of HIV-1 infection.

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