Induction of T-Cell Apoptosis by Human Herpesvirus 6

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The mechanisms of cell death in CD4$^+$ T cells mediated by human herpesvirus 6 (HHV-6) were investigated. The frequency of cell death in the human CD4$^+$ T-cell line JJHAN, which had been inoculated with HHV-6 variant A or B, appeared to be augmented by tumor necrosis factor alpha (TNF-α). Agarose gel electrophoresis of DNA from HHV-6-inoculated cells showed DNA fragmentation in multiples of the oligonucleosome length unit. The degree of DNA fragmentation increased when HHV-6-inoculated cells were cultured in the presence of TNF-α. Flow cytometry and Scatchard analysis of TNF receptors revealed an increase in the number of the p55 form of TNF receptors on JJHAN cells after HHV-6 inoculation. It also appeared that treatment with anti-Fas monoclonal antibody (MAb) induced marked apoptosis in HHV-6-inoculated cells. Transmission electron microscopy showed characteristics of apoptosis, such as chromatin condensation and fragmentation of nuclei, but virus particles were hardly detected in apoptotic cells. Two-color flow cytometric analysis using anti-HHV-6 MAb and propidium iodide revealed that DNA fragmentation was present predominantly in uninfected cells but not in productively HHV-6-infected cells. In addition, JJHAN cells incubated with UV light-irradiated and ultracentrifuged culture supernatant of HHV-6-infected cells appeared to undergo apoptosis. The present study demonstrated that both HHV-6 variants A and B induce apoptosis in CD4$^+$ T cells by indirect mechanisms, as reported recently in human immunodeficiency virus type 1 infection.

Human herpesvirus 6 (HHV-6) was first isolated from patients with lymphoproliferative disorder and AIDS (38). Subsequent studies have revealed that HHV-6 has tropism mainly to CD4$^+$ T cells (39) but can also infect B cells (1), CD8$^+$ T cells (25), and natural killer (NK) cells (29). This virus is now established as the causative agent of exanthem subitum (46) and is possibly associated with other various illnesses, including hepatitis, pneumonitis, lymphadenitis, and infectious mononucleosis-like disease (34). To clarify the pathogenesis of HHV-6 infection, it is essential to study the biological effects of the virus on the functions and characteristics of host cells. It has been reported recently that HHV-6 has modulatory effects on the expression of various cell surface molecules which are essential for implementation of cellular functions. That is, the expression level of the CD3–T-cell receptor complex markedly declines (12, 28) and de novo expression of CD4 is induced in CD4$^-$ T cells and NK cells following infection with HHV-6 variant A (25, 27, 29). These alterations of surface molecule expression result in dysfunction of T cells (12) and predispose CD4$^-$ T cells and NK cells to infection with human immunodeficiency virus type 1 (HIV-1) (25, 27, 29). In addition, it has been reported that HHV-6 and HIV-1 can coinfect CD4$^+$ T cells, resulting in *trans* activation of the HIV-1 long terminal repeat and acceleration of cell death (26). These data suggest that HHV-6 infection might contribute to the development of immunodeficiency in HIV-1-infected individuals and also directly cause the immunodeficient status. In the present study, to further study the biological effects of HHV-6 on CD4$^+$ T cells, we attempted to clarify the mechanisms of cell death in CD4$^+$ T cells mediated by HHV-6.

It has been proposed that apoptosis is involved in the pathogenesis of infections by various viruses, including adenovirus (35), Sindbis virus (23), influenza virus (41), lymphocytic choriomeningitis virus (36), vesicular stomatitis virus (22), measles virus (10), cytomegalovirus (21), chicken anemia virus (17), human T-lymphotropic virus type 1 (45), Moloney murine leukemia virus (37), feline immunodeficiency virus (32), and, most importantly, HIV-1 (3, 13, 14, 43). In the present study, we investigated the mechanisms of cell death of CD4$^+$ T cells mediated by a newly defined CD4$^+$ T-lymphotropic virus, HHV-6, focusing on the effect of tumor necrosis factor alpha (TNF-α), which is produced in various viral infections and known to be a functional modifier for various types of cell. We also studied the effect of stimulation through Fas on apoptosis of HHV-6-infected cells. Consequently, it was found that HHV-6 is an inducer of apoptosis in CD4$^+$ T cells and that the degree of apoptosis is augmented by TNF-α and anti-Fas monoclonal antibody (MAb). In addition, it appeared that apoptosis occurred predominantly in uninfected "bystander" cells but not in productively HHV-6-infected cells. The present data provide new insight into the pathogenesis of HHV-6 infection and its pathological role in diseases showing CD4$^+$ T lymphocytopenia, especially AIDS.

MATERIALS AND METHODS

Viruses and cells. The U1102 strain of HHV-6 variant A (7) and the Z29 strain of HHV-6 variant B (24) were grown in cord blood lymphocytes, which were stimulated with phytohemagglutinin (Difco, Detroit, Mich.) and cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO). When the cytopathic effects were maximal, HHV-6-infected cord blood lymphocytes were sonicated and centrifuged to remove the cell debris. The supernatants were used for virus inoculation. Uninfected cord blood lymphocytes were similarly cultured and treated as HHV-6-infected cells, and the supernatant of uninfected cells was used for mock infection. In some experiments, supernatant of HHV-6-infected cord blood cells and sonicated HHV-6-infected cells, both of which had been irradiated with UV light to inactivate HHV-6 and ultracentrifuged (131,000 × g for 2 h) to remove virion particles, were used. The CD4$^+$ human T-cell line JJHAN (42) was grown in RPMI 1640 medium supplemented with 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10% FCS.

Effects of TNF-α on cell death. JJHAN cells were inoculated with HHV-6 at a multiplicity of infection of approximately 0.1 50% tissue culture infective dose and cultured for 4 days. Virus-inoculated or mock-infected cells (2 × 10^6) were

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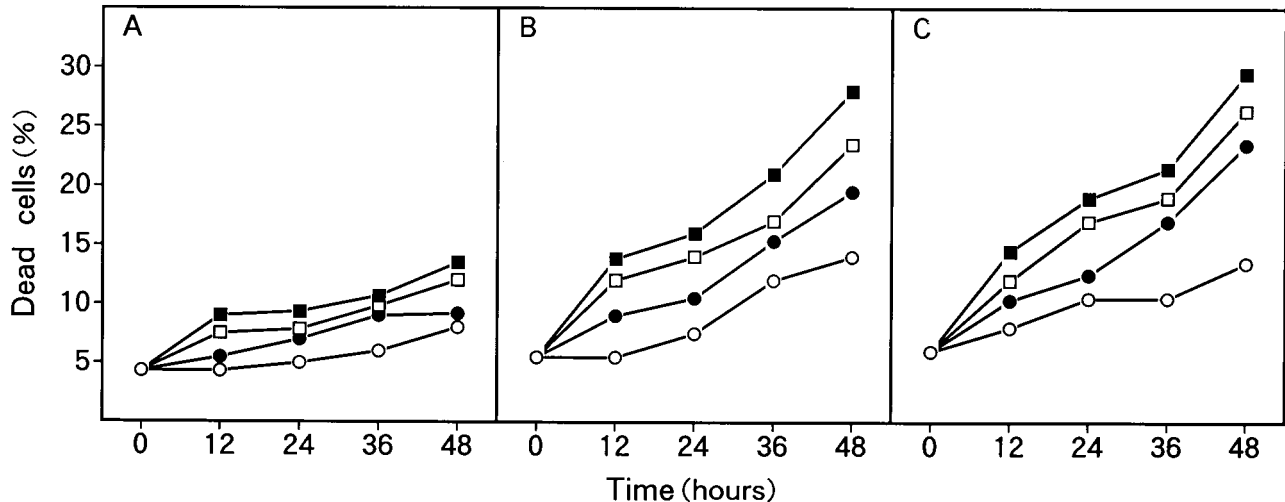
FIG. 1. Cytocidal effect of TNF-α on HHV-6-inoculated cells. Mock-infected (A), HHV-6 variant A (U1102)-inoculated (B), and HHV-6 variant B (Z29)-inoculated (C) JJHAN cells were cultured without (○) or with TNF-α at concentrations of 1 (●), 10 (□), and 100 (■) U/ml. Cell viability was determined by trypan blue exclusion. Each point is the average of triplicate determinations.

then suspended in 2 ml of RPMI 1640 medium supplemented with 10% FCS and cultured in a 16-mm-diameter well with or without TNF-α (Dainippon Pharmaceutical Co., Tokyo, Japan) at various concentrations for various periods. Cell viability was determined by the trypan blue dye exclusion test. Each sample was counted twice, and 500 or more cells were counted each time.

Transmission electron microscopy. JJHAN cells which had been inoculated with HHV-6 and treated with TNF-α were fixed with 2.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide, and gradually dehydrated. Samples were embedded in Epon 812, sectioned, stained with uranyl acetate and lead citrate, and examined with an H-800 microscope (Hitachi, Ibaragi, Japan).

Flow cytometric analysis of cell surface and intracytoplasmic molecules. Cell surface expression of the 55-kDa TNF receptor (TNF-R1) and the 75-kDa TNF receptor (TNF-R2) was examined by indirect immunofluorescence using MAbs htr-9 and utr-1 (4), kindly provided by M. Brockhaus, Hoffmann-La Roche, respectively. Fas expression on the cell surface was examined by direct immunofluorescence with the fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) isotype of anti-Fas MAb UB2 (MBL, Nagoya, Japan). Intracytoplasmic expression of oncoprotein Bcl-2 was examined by cell collection and centrifugation. The cell pellet was suspended in 1 ml of 0.25% paraformaldehyde and incubated for 20 min at room temperature. After being washed with phosphate-buffered saline (PBS), the cells were suspended in 1 ml of cold 70% methanol and incubated for 1 h at 4°C. After being washed with PBS, the cells were stained with FITC-conjugated anti-Bcl-2 MAb (DAKO, Glostrup, Denmark) or FITC-conjugated negative control mouse IgG1. The stained cells were then analyzed with an Epics profile flow cytometer (Coulter Electronics, Hialeah, Fla.).

DNA extraction and electrophoresis. Cells were collected from wells and centrifuged. The pellets were resuspended in 5 mM Tris-HCl (pH 7.4) containing 0.5% sodium dodecyl sulfate, 2 mM EDTA, and 0.5 mg of proteinase K per ml and incubated for 1 h at 50°C. RNase (50 μg) was then added, and incubation at 50°C was continued for 1 h. DNA was extracted from the resulting viscous solution with phenol-chloroform, precipitated with ethanol, dried, and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The extracted DNAs were subjected to electrophoresis in a 1.8% agarose gel and visualized by staining with ethidium bromide.

TNF-α binding assay. For measurement of TNF-α binding on the cell surface, a microassay for the binding of radiolabeled ligands to the cells (44) was applied. Briefly, cells (2×10^6) suspended in 20 μl of RPMI 1640 medium supplemented with 10% FCS in an Eppendorf tube were incubated with 100 μl of various concentrations of ^{125}I-labeled recombinant human TNF-α (New England Nuclear, Boston, Mass.) in the presence or absence of a 100-fold excess of unlabeled TNF-α for 1 h at 4°C in a total volume of 120 μl. Thereafter, the cell suspensions were overlaid with 500 μl of 20% sucrose and microcentrifuged at $1,400 \times g$ for 5 min. Each tube was quickly frozen in dry ice and then cut through the lower part of the sucrose layer. The radioactivity of the tip containing the cell pellet was counted with a gamma counter. Specific binding of TNF-α was calculated by subtracting the values of binding obtained in the presence of a 100-fold excess of the unlabeled TNF-α from the value of binding obtained with the labeled TNF-α alone. The data presented are average values for triplicate samples. Variation among triplicate samples was, in all cases, less than 10% of the average. The

number of TNF-α binding sites per cell and the dissociation constant were determined by Scatchard analysis.

Stimulation of Fas by anti-Fas MAb. HHV-6-inoculated or mock-infected JJHAN cells (2×10^6) suspended in 2 ml of RPMI 1640 medium supplemented with 10% FCS were cultured in a 16-mm-diameter well with or without the IgM isotype of anti-Fas MAb CH-11 (47) (MBL) at a concentration of 100 ng/ml for 36 h. DNAs were then extracted from each of the samples and subjected to electrophoresis as described above.

Flow cytometric analysis of DNA content. The percentage of apoptotic cells was measured by flow cytometric analysis of DNA stained with propidium iodide (PI) as described previously (31). DNA staining with PI in hypotonic buffer was performed with a CycleTEST PLUS DNA Reagent Kit (Becton-Dickinson, San Jose, Calif.) by following the manufacturer's protocol. The PI fluorescence of individual nuclei was measured with an Epics profile flow cytometer. Cell debris was gated out from the analysis by raising the light scatter threshold. The percentage of apoptotic cells was determined by measuring the hypodiploid DNA peak. To determine whether hypodiploid DNA was detectable in productively HHV-6-infected cells or uninfected cells, two-color flow cytometric analysis was performed as follows. After treatment with fixation buffer, the cells were incubated with anti-HHV-6 MAb OHV-1 (33) on ice for 30 min. The cells were then washed with PBS and incubated with FITC-conjugated goat anti-mouse IgG (Cappel, West Chester, Pa.) on ice for an additional 30 min. After being washed with PBS, the anti-HHV-6 MAb-treated cells were stained with PI as described above.

RESULTS

Death of HHV-6-inoculated cells cultured in the presence or absence of TNF-α. Figure 1 shows the time course of death of $CD4^+$ T cells, JJHAN cells, which were inoculated with HHV-6 variant A, U1102, or variant B, Z29, and cultured in the presence or absence of various concentrations of TNF-α, determined by trypan blue dye exclusion. The proportions of HHV-6 antigen-positive cells on day 4 of HHV-6 U1102 inoculation before TNF-α treatment and after 36 h of TNF-α treatment, determined by indirect immunofluorescence using anti-HHV-6 MAb, were 35 and 54%, respectively. On the other hand, those of HHV-6 Z29-inoculated cells were 5 and 6%, respectively. TNF-α showed little effect on the death of uninfected and mock-infected JJHAN cells at the concentrations employed, 1 to 100 U/ml. The proportion of dead cells among HHV-6-inoculated cells cultured without exogenous cytokines increased slightly during 48 h of culture, and the frequency of death in HHV-6-inoculated cells was augmented by the addition of TNF-α in a dose-dependent manner.
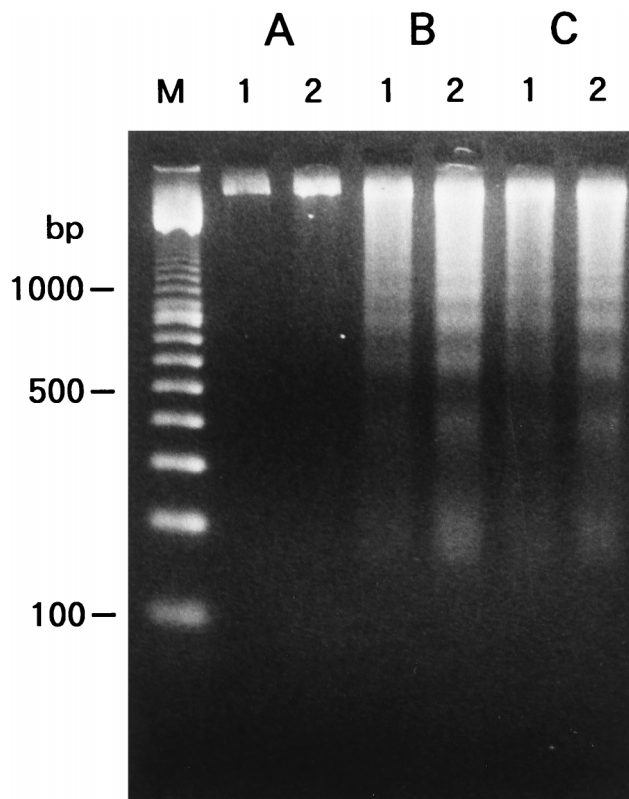
FIG. 2. DNA fragmentation of HHV-6-inoculated cells mediated by TNF-α. DNAs were extracted from mock-infected (A), HHV-6 variant A (U1102)-inoculated (B), and HHV-6 variant B (Z29)-inoculated (C) JJHAN cells which had been cultured without (lanes 1) or with (lanes 2) TNF-α (100 U/ml). DNAs were electrophoresed in 1.8% agarose gels and visualized with ethidium bromide. Lane M contained molecular weight markers.

DNA fragmentation of HHV-6-inoculated cells untreated and treated with TNF-α. We next addressed the question of whether apoptosis is induced in T cells by HHV-6 infection and performed agarose gel electrophoresis of DNA from HHV-6-inoculated cells cultured with or without TNF-α. As shown in Fig. 2, the ladder pattern of DNA on agarose gel electrophoresis, which reflects DNA fragmentation into oligonucleosome units, was scarcely detected in DNA from HHV-6-inoculated JJHAN cells cultured in the absence of exogenous cytokines. On the other hand, DNA from HHV-6-inoculated cells cultured in the presence of TNF-α was markedly fragmented into multiples of oligonucleosome length units. DNA fragmentation by TNF-α was detected in both HHV-6 variant A- and B-inoculated cells. On the other hand, DNA fragmentation was hardly detected in mock-infected cells cultured with TNF-α at the concentrations used in the present study, 1 to 100 U/ml. These data suggested that both HHV-6 variants A and B rendered JJHAN cells susceptible to TNF-α-induced apoptosis. The possibility that susceptibility to apoptosis was mediated by endogenous TNF-α produced by HHV-6 infection was unlikely, since enzyme-linked immunosorbent assay and reverse transcription-PCR analysis showed that TNF-α was not produced by JJHAN cells following inoculation with HHV-6 (data not shown). We also examined whether DNA of peripheral blood CD4+ T cells was fragmented into multiples of oligonucleosome length units by HHV-6 infection and treatment with TNF-α as was that of HHV-6-inoculated JJHAN cells. Consequently, an apparent ladder pattern of DNA on agarose gel

electrophoresis was detected, although the degree of DNA fragmentation in peripheral blood CD4+ T cells was relatively weak in comparison with that in JJHAN cells (data not shown). Therefore, further studies were performed with a human CD4+ T-cell line, JJHAN.

Morphologic changes in HHV-6-inoculated cells cultured with TNF-α. Apoptosis is morphologically characterized by condensation of nuclear chromatin, shrinkage and blebbing of the cytoplasm, and formation of apoptotic bodies. On the basis of these features, we examined the morphologic changes in HHV-6-inoculated cells cultured in the presence of TNF-α by transmission electron microscopy. Figure 3A shows transmission electron micrographs of the cells undergoing apoptosis. The ultrastructural changes in these cells included nuclei with marginalized and condensed chromatin matrix, fragmented nuclei, and intact mitochondria, i.e., the typical features of apoptosis. It was noteworthy that no virus particles were present in the apoptotic cells, whereas many viruses were detected predominantly in cells whose chromatin matrix was not condensed and mitochondria were undergoing degeneration (Fig. 3B and C).

Expression of TNF receptors. To investigate the mechanisms of apoptosis in HHV-6-inoculated T cells mediated by TNF-α, we next examined whether HHV-6 inoculation resulted in an increase of TNF receptor expression. Figure 4 shows flow cytometric analysis of the 55-kDa (p55) and 75-kDa (p75) forms of TNF receptors TNF-R1 and TNF-R2 on HHV-6 variant A-inoculated and mock-infected JJHAN cells. JJHAN cells appeared to express the p55, but not the p75, form of TNF receptor on their surfaces. As shown in Fig. 4, the expression level of the p55 form of TNF receptor on JJHAN cells increased slightly following inoculation with HHV-6 variant A. An increase in the expression of the p55 form of TNF receptor on JJHAN cells was similarly detected after inoculation with HHV-6 variant B. The mean fluorescence intensities of TNF-R1 on mock-infected, HHV-6 variant A-inoculated, and HHV-6 variant B-inoculated JJHAN cells were 7.7, 13.2, and 13.6, respectively. The kinetics study revealed that the increase in TNF receptor expression was detectable after 48 h of HHV-6 inoculation and that expression of the p75 form of TNF receptor was not induced by HHV-6 inoculation during 7 days of culture (data not shown).

The increase of TNF receptor expression on JJHAN cells after HHV-6 inoculation was confirmed by Scatchard analysis of TNF-α binding. Figure 5 shows representative data from three experiments for the Scatchard plot of ^{125}I-labeled TNF-α binding to HHV-6 variant A-inoculated and mock-infected cells. The number of TNF-α binding sites per cell given by the abscissa intercept was significantly higher in HHV-6-inoculated cells than in mock-infected cells, 1,445 per cell and 758 per cell, respectively. On the other hand, the dissociation constants for TNF-α were not significantly different for HHV-6-inoculated and mock-infected cells, 2.9×10^{-10} and 2.4×10^{-10} M, respectively. These data indicated that HHV-6 inoculation of JJHAN cells induced an increase in the number of TNF receptors expressed on the cell surface and did not affect the affinity of TNF-α binding.

Apoptosis mediated by anti-Fas MAb. In recent years, a number of molecules regulating apoptosis have been identified. Among them, Fas and Bcl-2 are now recognized to be important molecules that induce and inhibit apoptosis, respectively. We first compared the levels of Fas expression on HHV-6-inoculated and mock-infected JJHAN cells. Flow cytometric analysis revealed that Fas was expressed weakly on JJHAN cells and that its level of expression did not differ significantly between HHV-6-inoculated and mock-infected JJHAN cells
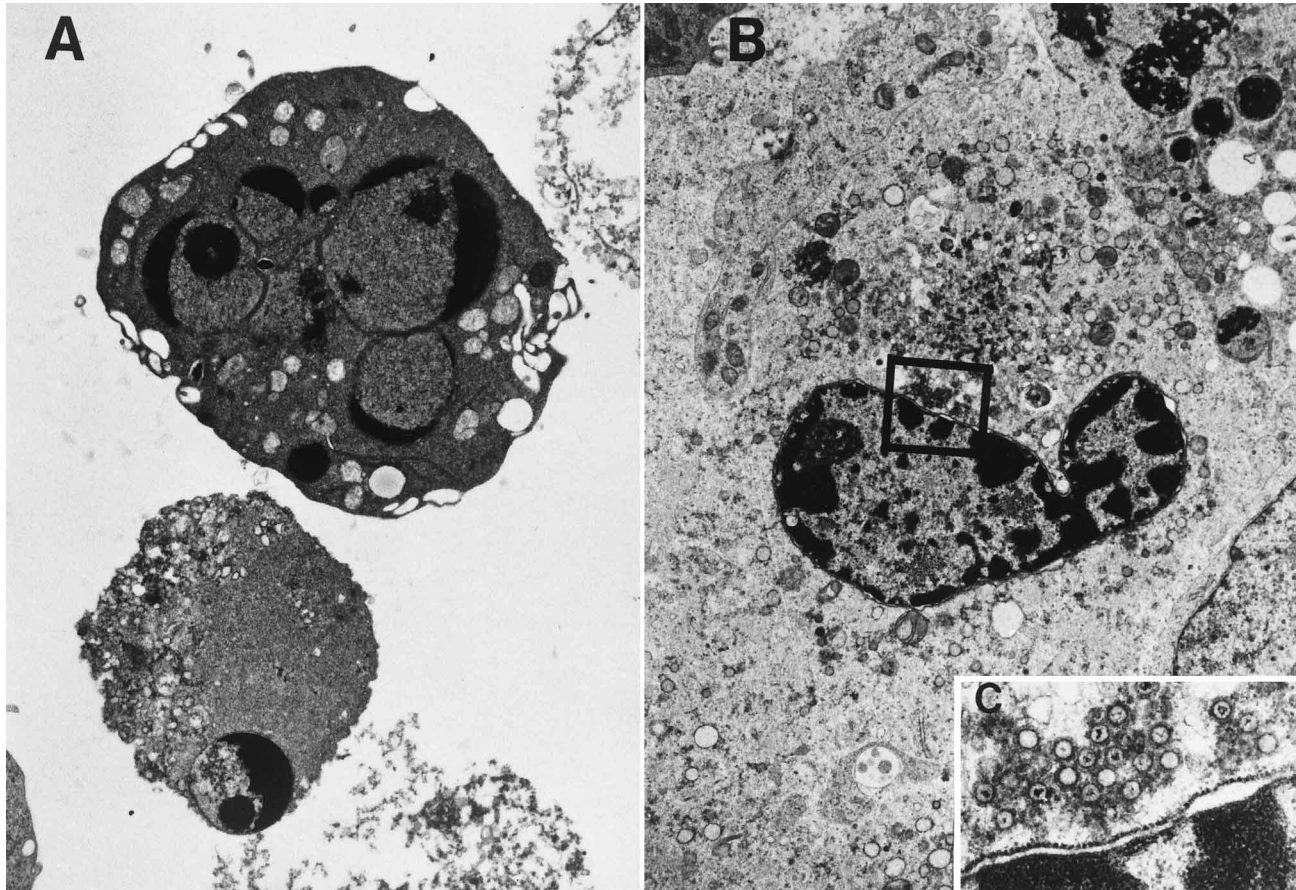
FIG. 3. Transmission electron micrograph of apoptosis in HHV-6-inoculated cells. HHV-6 variant A (U1102)-inoculated JJHAN cells were cultured with TNF-α at a concentration of 100 U/ml for 36 h. Note that no HHV-6 particles are detectable in the apoptotic cells (A) (magnification, ×5,600), whereas many HHV-6 particles are present in the cells showing no features of apoptosis (B) (magnification, ×5,600). At a higher magnification (×26,100) of the boxed area of panel B, HHV-6 particles were identified in the cytoplasm of the cell (C).

(data not shown). Similarly, the level of Bcl-2 expression in the cytoplasm appeared to be unchanged following inoculation with HHV-6 (data not shown). Flow cytometry was performed repeatedly during incubation for 12 to 96 h, but no significant change in Fas and Bcl-2 expression levels was detected at any time. It is known that stimulation of Fas by anti-Fas MAb mediates apoptosis (47). On the basis of this finding, we next examined the apoptosis sensitivity of HHV-6-inoculated and mock-infected cells to stimulation with anti-Fas MAb. As shown in Fig. 6, DNA fragmentation into multiples of oligo-nucleosome length units was scarcely detected in anti-Fas MAb-stimulated, mock-infected JJHAN cells. On the other hand, the degree of DNA fragmentation induced by anti-Fas MAb was markedly augmented in HHV-6 variant A- and B-inoculated cells.

Flow cytometric analysis of apoptotic cells. The augmentation of DNA fragmentation in HHV-6-inoculated cells by TNF-α and anti-Fas MAb was confirmed by flow cytometric analysis of DNA stained with PI. Representative data are shown in Fig. 7. As observed on DNA electrophoresis, little increase in the number of nuclei with hypodiploid DNA content was detected in mock-infected JJHAN cells incubated with TNF-α or anti-Fas MAb. On the other hand, the percentage of the hypodiploid DNA peak in HHV-6-inoculated cells was increased by TNF-α and anti-Fas MAb, as shown by electrophoresis. Representative percentages of hypodiploid DNA

in HHV-6 variant A-inoculated JJHAN cells which were unstimulated, incubated with TNF-α, or stimulated with anti-Fas MAb were 9.0, 20.8, and 21.6%, respectively. Similarly, those in HHV-6 variant B-inoculated cells were 10.3, 23.1, and 22.0%, respectively.

Detection of DNA fragmentation in HHV-6-infected and uninfected cells. We addressed the question of whether apoptosis occurred in productively HHV-6-infected cells or cells in which the virus did not replicate. As shown in Fig. 8, a hypodiploid DNA peak was detected predominantly in HHV-6 antigen-negative cells and most of the DNA content of HHV-6 antigen-positive cells was not hypodiploid. These data strongly suggested that apoptosis occurred predominantly in the cells in which HHV-6 did not replicate. To confirm this, we separated HHV-6 antigen-positive and -negative cells by using anti-HHV-6 MAb and anti-mouse IgG-coated magnetic beads and performed agarose gel electrophoresis of DNA from each sample. The apparent ladder pattern on gel electrophoresis was detectable in DNA from HHV-6 antigen-negative cells but not in that from HHV-6 antigen-positive cells (data not shown).

Effects of UV-irradiated and ultracentrifuged supernatant of HHV-6-infected cells on induction of apoptosis. To further verify the possibility that acquisition of susceptibility to apoptosis does not require HHV-6 entry and replication, the effects of sonicated HHV-6-infected cells or culture supernatant of HHV-6-infected cells which had been irradiated with UV to
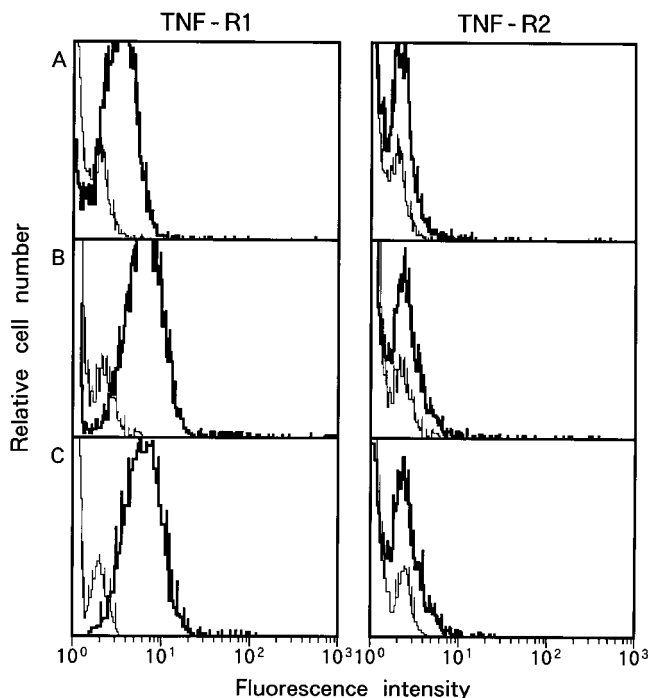
FIG. 4. Flow cytometric analysis of TNF receptors. Mock-infected (A), HHV-6 variant A (U1102)-inoculated (B), and HHV-6 variant B (Z29)-inoculated (C) JJHAN cells were stained by indirect immunofluorescence with anti-p55 TNF receptor MAb or anti-p75 TNF receptor MAb and then with FITC-conjugated goat anti-mouse IgG. Staining with control mouse IgG and FITC-conjugated goat anti-mouse IgG is shown as thin lines.

FIG. 6. DNA fragmentation of HHV-6-inoculated cells mediated by anti-Fas MAb. DNAs were extracted from mock-infected (A), HHV-6 variant A (U1102)-inoculated (B), and HHV-6 variant B (Z29)-inoculated (C) JJHAN cells which had been cultured without (lanes 1) or with (lanes 2) the IgM isotype of the anti-Fas MAb (100 ng/ml) for 36 h. DNAs were electrophoresed in 1.8% agarose gels and visualized with ethidium bromide. Lane M contained molecular weight markers.

inactivate HHV-6 and ultracentrifuged to remove virion particles on induction of apoptosis were examined. UV-irradiated HHV-6 was unable to replicate in the cells, and no infectious HHV-6 was detected in the ultracentrifuged materials by cytopathic effect detection and immunofluorescence assay. As shown in Fig. 9, JJHAN cells which had been cultured with sonicated, UV irradiated, and ultracentrifuged HHV-6-infected cells and those which had been cultured with UV-irra-
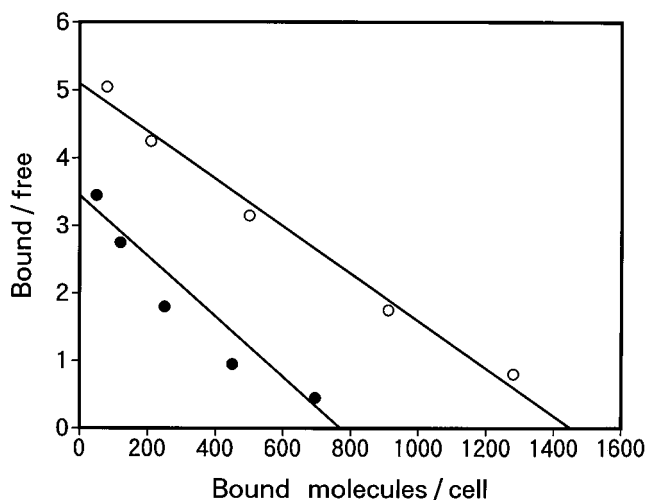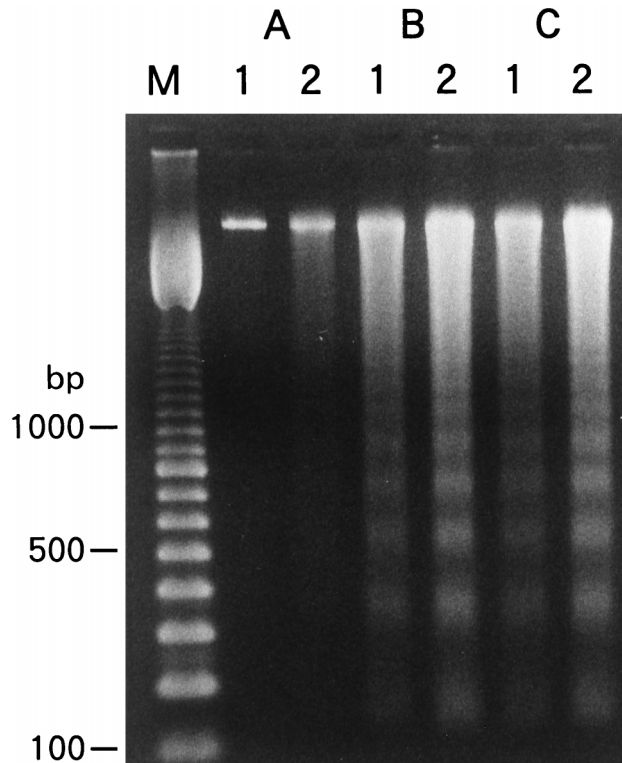

FIG. 5. Scatchard analysis of TNF-α binding on HHV-6 variant A (U1102)-inoculated (○) and mock-infected (●) JJHAN cells. Each symbol represents the mean of triplicate data points.

diated and ultracentrifuged supernatant of HHV-6-infected cells both underwent apoptosis in the presence of TNF-α. Representative percentages of hypodiploid DNA in these cells were 20.3 and 19.3%, respectively. On the other hand, no apparent apoptosis was detected in JJHAN cells cultured with sonicated, mock-infected cells and those cultured with supernatant of mock-infected cells. In addition, it appeared that sonicated, HHV-6-infected cells and culture supernatant of HHV-6-infected cells which had been treated with anti-HHV-6 serum and ultracentrifuged had the ability to render T cells susceptible to apoptosis (data not shown). These data strongly suggest that entry of HHV-6 into and replication of HHV-6 in the cells are not required for induction of susceptibility to apoptosis.

DISCUSSION

The present study was undertaken to investigate the mechanisms of cell death in CD4$^+$ T cells induced by infection with a newly defined CD4$^+$ T-lymphotropic herpesvirus, HHV-6. The new findings obtained from the present series of experiments were as follows. First, HHV-6 inoculation alone weakly induced apoptosis of the human CD4$^+$ T cell line JJHAN with no exogenous cytokine or stimulation. Second, the degree of apoptosis in HHV-6-inoculated CD4$^+$ T cells was augmented by TNF-α. Third, the number of p55 form TNF receptors expressed on JJHAN cells increased following inoculation with HHV-6. Fourth, although HHV-6 inoculation did not affect the level of Fas expression, it rendered JJHAN cells more
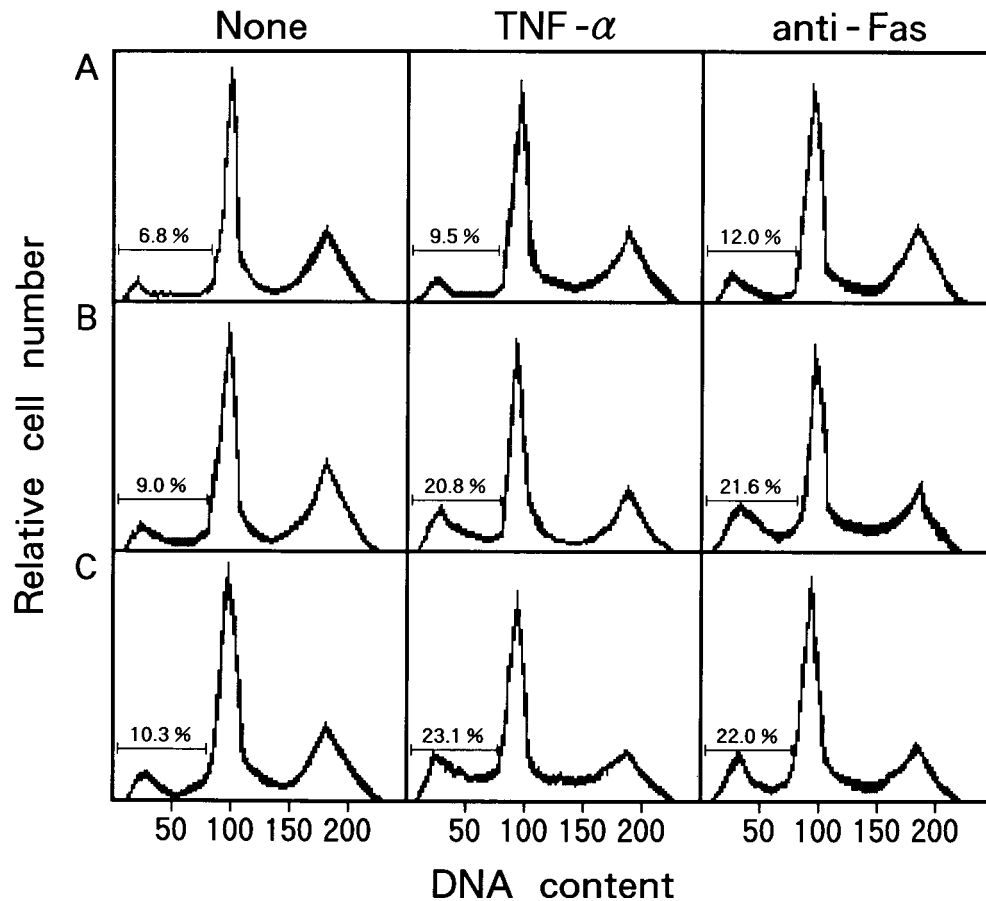
FIG. 7. Flow cytometric DNA fluorescence profiles. Mock-infected (A), HHV-6 variant A (U1102)-inoculated (B), and HHV-6 variant B (Z29)-inoculated (C) JJHAN cells were cultured with or without TNF-α (100 U/ml) or anti-Fas MAb (100 ng/ml) for 36 h. Nuclei were stained with PI, and the DNA contents of the cells were analyzed by flow cytometry. The values above the bars are percentages of apoptotic nuclei.

susceptible to apoptosis upon stimulation with anti-Fas MAb. Fifth, apoptosis occurred predominantly in HHV-6 antigen-negative cells, and UV-irradiated HHV-6 virion-free supernatant of HHV-6-infected cells was able to induce susceptibility

to apoptosis, suggesting that HHV-6 entry and replication are not required for induction of apoptosis in T cells.

It was found that expression of the p55 form of TNF receptor on JJHAN cells was up-regulated by HHV-6 inoculation
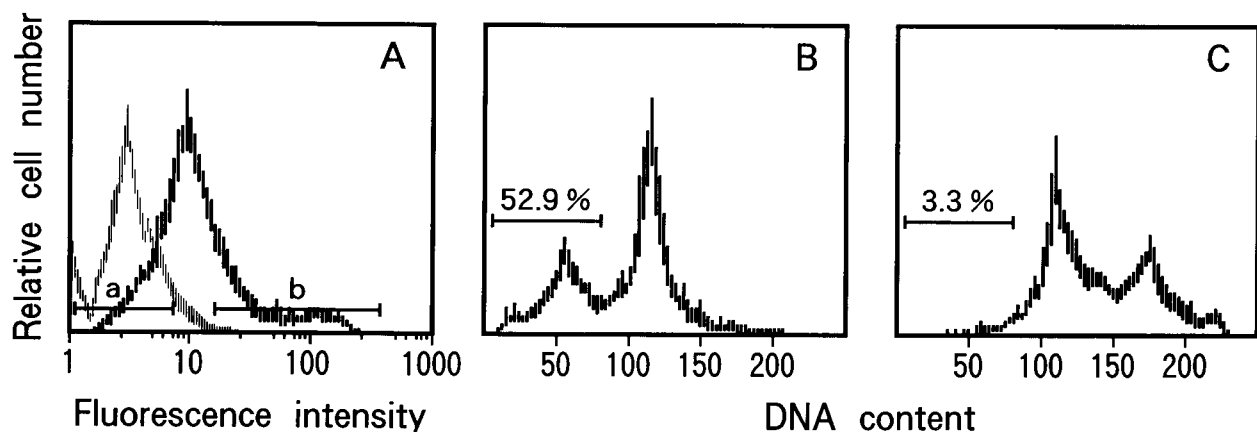

FIG. 8. DNA fragmentation of HHV-6 antigen-positive and -negative cells. JJHAN cells which had been inoculated with HHV-6 variant A (U1102) and treated with TNF-α were stained with anti-HHV-6 MAb and FITC-conjugated goat anti-mouse IgG (thick line) or with FITC-conjugated goat anti-mouse IgG alone (thin line in panel A). The DNA content of HHV-6 antigen-negative cells gated in area a of panel A (B) and that of HHV-6 antigen-positive cells gated in area b of panel A (C) were analyzed by flow cytometry. The values above the bars are percentages of apoptotic nuclei.
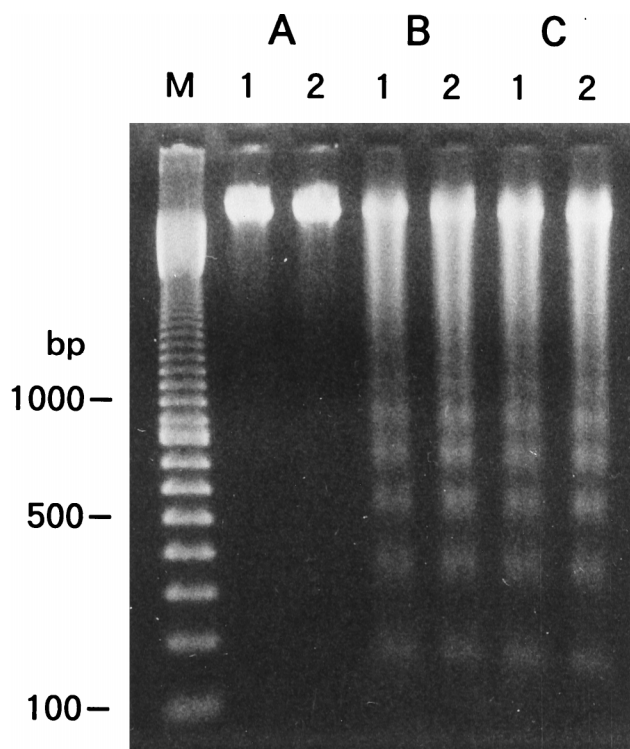
FIG. 9. DNA fragmentation of T cells incubated with sonicated HHV-6-infected cells or culture supernatant of HHV-6-infected cells which had been UV irradiated and ultracentrifuged. JJHAN cells which had been cultured with sonicated, UV-irradiated, and ultracentrifuged mock-infected cells (A, lane 1); UV-irradiated and ultracentrifuged culture supernatant of mock-infected cells (A, lane 2); sonicated, UV-irradiated, and ultracentrifuged HHV-6 variant A (U1102)-infected cells (B, lane 1); UV-irradiated and ultracentrifuged culture supernatant of HHV-6 variant A (U1102)-infected cells (B, lane 2); sonicated, UV-irradiated, and ultracentrifuged HHV-6 variant B (Z29)-infected cells (C, lane 1); or UV-irradiated and ultracentrifuged supernatant of HHV-6 variant B (Z29)-infected cells (C, lane 2) for 3 days were cultured with TNF-α (100 U/ml) for 36 h. The DNAs were electrophoresed in 1.8% agarose gels and visualized with ethidium bromide. Lane M contained molecular weight markers.

and that the frequency of apoptosis in HHV-6-inoculated cells was augmented by TNF-α. The activity of TNF-α is induced after binding to either of two distinct cell surface receptors, the p55 and p75 forms, which are expressed independently on a variety of cell types (4). The intracellular domain of the p55 TNF receptor contains a "death domain" similar to that found in the intracellular domain of Fas, while the p75 TNF receptor does not contain this domain (16). Therefore, the increase in the number of p55 TNF receptors induced by HHV-6 inoculation may be one of the causes of the susceptibility of HHV-6-inoculated cells to TNF-α-mediated apoptosis. However, since the degree of increase in TNF receptor expression induced by HHV-6 inoculation was slight, it is unlikely that only up-regulation of the TNF receptor is the cause of TNF-α-mediated apoptosis. Therefore, it should be considered that some intracellular molecules which render cells susceptible to TNF-α-mediated apoptosis might be induced or activated by HHV-6 infection.

Although it is well known that apoptosis is mediated by various viral infections, the precise mechanisms of apoptotic death in virus-infected cells are unclear. Various molecules which regulate apoptosis have been identified, and their roles in virus-induced apoptosis have been extensively investigated. Among them, Fas (CD95), a member of the TNF-nerve growth factor receptor superfamily, is known to play an important role in induction of apoptosis in transformed cell lines and activated T cells. It has been reported that Fas expression on T cells is augmented by HIV-1 infection and that stimulation of Fas by anti-Fas MAb induces apoptosis of a chronically HIV-1-infected T-cell line and CD4+ and CD8+ peripheral blood T cells of HIV-1-infected individuals (18, 20). Augmentation of Fas expression and susceptibility to apoptosis induced by anti-Fas MAb have also been reported in influenza virus-infected cells (40). These findings suggest that the Fas-Fas ligand system might also be involved in the apoptosis of HHV-6-inoculated cells. Accordingly, we investigated whether Fas expression and anti-Fas MAb-mediated apoptosis of T cells was augmented by HHV-6 inoculation. Consequently, it appeared that there was no significant difference between the levels of Fas expression in HHV-6-inoculated and mock-infected cells, although stimulation with anti-Fas MAb mediated apoptosis selectively in HHV-6-inoculated cells. These data strongly suggest that HHV-6 affects the signaling system through Fas and renders host cells susceptible to apoptosis upon stimulation of Fas. Taken together with our present finding that TNF-α also mediates apoptosis of HHV-6-inoculated cells, it can be considered that a common pathway of apoptosis is present in signaling systems involving the TNF receptor and Fas, as suggested recently (8), and that HHV-6 may activate this pathway. Although more detailed study is needed to clarify the roles of Fas in apoptosis of HHV-6-inoculated cells, our present findings suggest that the Fas-Fas ligand system is involved in the mechanism of apoptotic death in HHV-6-inoculated cells, as in HIV-1-infected cells.

In contrast to Fas, proto-oncogene *bcl-2* is known to be an inhibitor of apoptosis and is also thought to be involved in the mechanisms of death and survival of virus-infected cells. It has been reported that constitutive expression of *bcl-2* by gene transfection prevents apoptotic death of influenza virus-infected cells and human T-cell leukemia virus type I Tax-mediated apoptosis (15, 45). In addition, a correlation between the low level of Bcl-2 expression in T cells of patients with acute viral infections, such as infectious mononucleosis caused by Epstein-Barr virus and chickenpox resulting from varicella-zoster virus infection, and apoptosis of T cells in culture has been reported (2). A low level of Bcl-2 expression has also been reported in peripheral blood T cells of HIV-1-infected individuals and is considered to be one of the causes of their T-cell depletion (6). Our data, however, showed that expression of Bcl-2 was not affected by HHV-6 infection, suggesting that Bcl-2 was not involved in apoptosis of HHV-6-inoculated T cells.

It is of considerable importance to clarify whether HHV-6 replication in individual CD4+ T cells is essential for priming of the cells to undergo apoptosis. Peripheral blood lymphocytes in HIV-1-infected individuals are prone to apoptosis in response to stimulation of Fas, although this cannot account for the level of HIV-1-infected cells, since only a small proportion of peripheral blood lymphocytes are infected with HIV-1. In addition, it has been reported that treatment of CD4+ T cells with HIV-1 gp120 and anti-gp120 antibody primes them for activation-induced apoptosis (3). More recently, a study using in situ labeling of lymph nodes from HIV-1-infected children has demonstrated that DNA fragmentation is rarely observed in HIV-1-producing cells and that HIV-1 RNA is rarely detected in apoptotic cells (11). These findings suggest that viral replication is not necessary for virus-induced apoptosis. The following data from the present study strongly suggest that apoptosis occurred predominantly in HHV-6-uninfected cells and not in productively HHV-6-infected cells, as suggested recently for HIV-1-induced apopto-

sis. First, apoptosis was detected morphologically in small cells and not in virus-producing large syncytial cells, and virus structures were hardly detected in apoptotic cells by electron microscopy. Second, two-color flow cytometry using anti-HHV-6 MAb and PI staining and separation of HHV-6-inoculated cells into HHV-6 antigen-positive and -negative cells demonstrated that apoptosis occurred predominantly in HHV-6 antigen-negative cells. Third, HHV-6 virion-free supernatant of HHV-6-infected cells was able to render T cells susceptible to apoptosis. Although the precise mechanism of virus-induced apoptosis in uninfected cells is obscure, it can be hypothesized that a subcomponent of the HHV-6 structure, such as gp120 in HIV-1-induced apoptosis, and some kinds of cytokines produced by HHV-6-infected cells may render uninfected bystander cells susceptible to apoptosis. It can also be speculated that HHV-6 encodes viral gene products that inhibit cellular apoptosis and persists in macrophages and CD4$^+$ T cells in immunocompetent individuals and that replication of HHV-6 may induce apoptosis of uninfected T cells in immunocompromised patients.

The data presented here provide new insight into the pathogenesis of virus-induced T-cell immunodeficiencies, such as AIDS. Although previous data concerning the effect of HHV-6 on HIV-1 replication are controversial, it was reported that HHV-6 *trans* activated the HIV-1 long terminal repeat (9, 26, 30, 48). In addition, disseminated HHV-6 infection is frequently observed in patients with AIDS (5, 19), suggesting that HHV-6 is an important cofactor for the development of AIDS in HIV-1-infected individuals. Taken together with these previous reports, our present study provides new information on the role of HHV-6 in the development of AIDS in HIV-1-infected individuals.

ACKNOWLEDGMENTS

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