JOURNAL OF VIROLOGY, Dec. 1997, p. 9753–9763 Vol. 71, No. 12
0022-538X/97/$04.00+0

A Major Human Immunodeficiency Virus Type 1-Initiated Killing Pathway Distinct from Apoptosis

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Received 14 March 1997/Accepted 9 September 1997

We have investigated the relative contribution of apoptosis or programmed cell death (PCD) to cell killing during acute infection with T-cell-tropic, cytopathic human immunodeficiency virus type 1 (HIV-1), by employing diverse strategies to inhibit PCD or to detect its common end-stage sequelae. When Bcl-2-transfected cell lines were infected with HIV-1, their viability was only slightly higher than that of control infections. Although the adenovirus E1B 19-kDa protein has been reported to be a stronger competitor of apoptosis than Bcl-2, it did not inhibit HIV-mediated cell death better than Bcl-2 protein. Competition for Fas ligand or inactivation of the Fas pathway secondary to intracellular mutation (MOLT-4 T cells) also had modest effects on overall cell death during acute HIV infection. In contrast to these observations with HIV infection or with HIV envelope-initiated cell death, Tat-expressing cell lines were much more susceptible (200% enhancement) to Fas-induced apoptosis than controls and Bcl-2 overexpression strongly (75%) inhibited this apoptotic T-cell death. PCD associated with FasR ligation resulted in the cleavage of common interleukin-1β-converting enzyme (ICE)-protease targets, poly(ADP-ribose) polymerase (PARP) and pro-ICE, whereas cleaved products were not readily detected during HIV infection of peripheral blood mononuclear cells or T-cell lines even during periods of extensive cell death. These results indicate that one important form of HIV-mediated cell killing proceeds by a pathway that lacks the characteristics of T-cell apoptosis. Our observations support the conclusion that at least two HIV genes (*env* and *tat*) can kill T cells by distinct pathways and that an envelope-initiated process of T-cell death can be discriminated from apoptosis by many of the properties most closely associated with apoptotic cell death.

Human immunodeficiency virus (HIV) disease is characterized by a relentless decline in $CD4^+$ T cells, resulting in the development of AIDS (21). Immediately after infection and during late-stage progression to AIDS, $CD4^+$ T-cell loss is typically quite rapid. At least during progression to AIDS, viral isolates (T-cell-tropic [T-tropic]) are highly and directly cytopathic for $CD4^+$ T cells (70, 71). In contrast, viral isolates from the plateau phase of HIV disease, lasting on average 7 to 10 years, are less directly cytopathic for T cells (M-tropic) (12, 13). During this period, HIV disease is manifested by significant features of immune disregulation, including accelerated apoptosis in T cells (2, 24, 46) and other lymphocyte subsets (22).

The mechanisms of $CD4^+$ T-cell death during HIV disease are incompletely understood but might likely be multifactorial, depending on the stage of disease progression. Recent studies of patients with AIDS have shown that HIV replication and T-cell turnover in these individuals are much greater than previously expected, suggesting that direct viral cytopathicity could make a major contribution to $CD4^+$ T-cell decline during HIV disease (32, 75) HIV infection has further been reported to abnormally activate T cells and to damage the balance between TH1 and TH2 helper T cells (10, 19), leading to accelerated apoptosis and $CD4^+$ T-cell loss, although the amount of this apoptosis does not closely correlate with HIV disease progression (47). Several other abnormalities, including a selective destruction of memory T-helper cells by viral infection (60), T-cell suppression (17), clonal anergy, autoimmune reactions (56), and superantigen-induced cellular tolerance (34) have all been proposed to contribute to the development of AIDS.

Apoptosis is a physiological process which has a central role in tissue development and homeostasis (30, 54). Elegant histological studies first characterized apoptotic cells by their distinct morphological changes, which include plasma membrane ruffling and blebbing, nuclear shrinkage, and chromatin condensation (9). These changes are associated with the activation of a cellular nuclease (78), resulting in the fragmentation of DNA into a ladder of regular nucleosomal subunits (79). Thymic T cells, which undergo extensive apoptosis during their maturation to eliminate autoreactive precursors, have been one widely studied model of apoptosis (11). While the fundamental contribution of apoptosis to most if not all types of physiologic cell death, including T-cell tolerance induction, is now widely appreciated, its participation in pathological forms of cell death, such as that occurring during HIV type 1 (HIV-1) infection, is less fully understood and might vary depending on the particular pathological agent.

Important recent advances have greatly contributed to our understanding of the biochemical pathway activated during apoptosis or programmed cell death (PCD). Based on the paradigm first worked out in *Caenorhabditis elegans*, at least one antiapoptotic protein of the Bcl-2 family (28) and one

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cysteine protease of the interleukin-1β-converting enzyme (ICE) protease family (29, 84) appear to be nearly universal intracellular regulators of the apoptotic pathway. The precise identity of the individual apoptotic contributors may vary according to the cell type undergoing PCD, reflecting at least in part the particular cell surface receptor (R) ligated at the initiation of the apoptotic signal. These receptors triggering apoptosis are most commonly members of the tumor necrosis factor (TNF)R/FasR family of death domain proteins (51, 76). Despite some cell-type-specific variation in these regulators, apoptotic stimuli converge to activate the cleavage of ICE protease substrates, including poly(ADP-ribose) polymerase (PARP) and pro-ICE, and to alter cell structure, resulting in involution and apoptotic cell death.

The *Bcl-2* gene, originally isolated from a translocation on chromosome 18, common in B-cell lymphomas (5, 74), was discovered to be the first in a family of antiapoptotic proteins with sequence and functional homology to the *C. elegans Ced-9* gene (28). Several additional members of this family were subsequently isolated as proteins capable of dimerizing with Bcl-2 or its homologs, some of which, like Bcl-2, function to enhance cell survival, while others instead promote cell death (for reviews, see references 14, 25, and 81). The spectrum of Bcl-2 activity is partially defined by its cell-type-specific expression and partially defined by its interaction with some protagonistic family members, such as Bax, but not with others, such as Bak (81). The adenovirus E1B protein, a homolog of Bcl-2, is one of the strongest inhibitors of apoptosis yet characterized, blocking apoptosis and interacting with the apoptotic protagonists Bak as well as Bax (25, 81).

While ligation of several members of the death domain-containing, TNF receptor family can initiate apoptosis (35, 72, 82), the FasR (APO-1, CD95) is the most important for PCD in T cells. FasR is proximally linked to the intracellular apoptotic machinery via its direct interaction with the ICE protease, FLICE (7, 50). Engagement of the T-cell receptor on T-cell tumor lines, or triggering of normal T cells, induces expression of both Fas and its ligand (Fas-L), resulting in FLICE proteolytic activation and apoptosis by a process usually referred to as activation-induced cell death (AICD) (1, 16). Recently, it has been shown that circulating T lymphocytes from HIV-infected individuals have increased expression of FasR on their surfaces (38) and are more sensitive to Fas-induced apoptosis (20, 38), leading to a proposal that FasR may initiate the PCD observed during HIV infection.

In this report, we have designed several overlapping in vitro strategies to evaluate the participation of apoptosis in acute T-cell killing during infection with T-tropic, cytopathic HIV-1. Our results show that two antiapoptotic proteins, Bcl-2 and adenovirus E1B, only marginally decrease overall cell death in these cultures. Similarly, direct inhibition of FasR could not inhibit most HIV-induced cell death. Cleavage of PARP and GDP dissociation inhibitor for the Rho family (D4-GDI) proteins, two ICE/CED-3 family protease substrates commonly reflecting activation of the apoptotic cell death pathway, are not observed during HIV infections. D4-GDI normally functions to inhibit members of the Rho GTPase family and is a cytosolic substrate for ICE-like proteases. These data suggest that a major pathway of HIV-induced cell death proceeds by a nonapoptotic pathway. During the course of these studies, we have confirmed previous reports showing that HIV Tat functioned to potently enhance apoptotic T-cell death, possibly as proposed by upregulating FasR expression on T cells (20, 38, 76). This cell death has typical features of PCD and is inhibitable by Bcl-2 protein. Our studies suggest that at least two HIV genes (*env* and *tat*) induce distinct cell death pathways.

MATERIALS AND METHODS

Cell lines and cell cultures. $CD4^+$ Jurkat T cells, MOLT-4 cells, Jurkat T cells expressing the HIV envelope glycoproteins gp160, gp120, and gp41, as well as HIV-1 Tat and Rev proteins (J-env cells) (68), and other cell lines (Bcl-2, E1B, and Tat) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg of gentamicin/ml. J-env 120/41 cells (primarily used in this report) differ from J-env 160 cells insofar as the latter do not express any fully processed HIV envelope gp120 or gp41. Adenovirus E1B 19K-expressing cell lines were described elsewhere (3). Briefly, the cell lines were engineered by coelectroporation of Jurkat cells with plasmids pCMV19K or pMT19K and pSV2neo for selection. The Tat-expressing cell lines were established by transfection of Jurkat cells with a construct encoding a full-length HIV-1 (Hxb2) Tat.

Viral infection. A high-titer HIV-1 stock was prepared by passages of HIV-1 (LAI) on Jurkat cells. The T-tropic, laboratory strain LAI was originally isolated from an infected individual with AIDS. Supernatants were collected, and viral titer was assessed by p24 assay. Under routine experimental conditions, 2.5×10^6 to 5×10^6 Jurkat cells were inoculated with 100 μl of HIV-1(LAI) (2 to 4 ng of p24) in 0.5 ml of medium and incubated at 37°C in an atmosphere of 5% CO_2 with gentle agitation every 15 min for 2 h. Afterward the cells were washed twice and resuspended in complete medium, and cultures were monitored for viral replication by p24 assay. In some experiments, peripheral blood mononuclear cells (PBMC) were infected with HIV-1(G571) (syncytium inducing [SI] in PBMC).

Virus detection. HIV-1 p24 antigen production in cell supernatants was assessed by an enzyme immunoassay (HIV-1 p24 antigen capture assay kit; Coulter, Miami, Fla.).

Induction of HIV T-cell killing. To induce cell death, we have exploited the CD4-HIV envelope glycoprotein interaction (68). Briefly, 2×10^6 Jurkat T cells were mixed with 2×10^6 HIV envelope-expressing cells, centrifuged, resuspended in 1 ml of complete medium, and incubated at 37°C in 5% CO_2 for 1 h. Afterward, 9 ml of medium was added and the cells were incubated for various times, as indicated in the particular assay.

Fas induction of apoptosis. The antihuman Fas (CD95) monoclonal antibody CH-11 (Kamiya Biomedical Company, Thousand Oaks, Calif.) reacts specifically with human antigen and has cytolytic activity on human cells. CH-11 anti-Fas antibody was added to cultures (50 to 250 ng/ml), and cell viability was monitored by trypan blue exclusion or by fluorescence-activated cell sorter analysis. In some experiments the anti-human Fas antibody ZB4 was used to block the apoptosis-inducing activity of antihuman Fas CH-11.

Transfection of the human *Bcl-2* gene into Jurkat and HIV envelope cells. Jurkat and HIV envelope cells were cotransfected by electroporation with a selectable marker for hygromycin resistance and with either a spleen focus-forming virus (SFFV) control construct or an SFFV-Bcl-2-nl construct encoding the human Bcl-2 p26 protein (33). For electroporation, 5×10^6 cells were washed in ice-cold phosphate-buffered sucrose (272 mM sucrose, 7 mM $NaPO_4$ [pH 7.4], 1 mM $MgCl_2$), resuspended in 700 μl of ice-cold phosphate-buffered sucrose, and transferred to a chilled 0.4-cm cuvette containing 10 μg of SFFV construct and 2 μg of cotransfected plasmid with the selectable marker. The cells were kept on ice for 10 min prior to electroporation at 370 V, 25 μF, for Jurkat cells and 350 V, 25 μF, for HIV envelope cells. After a further 10 min on ice, the cells were diluted to concentrations of 10^3, 10^4, and 10^5 cells per 100 μl and transferred to 96 flat-bottom wells in medium without hygromycin B. After 48 h, 100 μl of medium containing 400 μg of hygromycin B/ml was added and the culture were maintained under hygromycin B selection thereafter.

Immunoblot analysis. Single-cell suspensions were lysed in 150 mM NaCl, 50 mM Tris HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg each of leupeptin and aprotinin/ml at 4°C for 30 min. Protein lysates were clarified by centrifugation, normalized for protein content with the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.) according to manufacturer's instructions, denatured by boiling in 3% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and fractioned by electrophoresis in nondenaturing discontinuous (5 to 15%) or continuous (15 or 11%) polyacrylamide gels (PAGE). Gels were electroblotted onto nitrocellulose filters, blocked in 3% BSA for 1 h at room temperature, and subsequently probed with antibodies. After incubation with primary antibodies for 3 h to overnight, specific proteins were detected with ^{125}I-protein A or incubated with secondary antibodies and detected by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.). The 6C8 monoclonal antibody to human Bcl-2 was described elsewhere (33). The anti-cyclin B polyclonal antibody (44) and the cdk1 (p34cdc2) antibody, specific for the C terminus (18), were raised in rabbits. The phycoerythrin-labeled Jo2 anti-Fas antibody was purchased from PharMingen (San Diego, Calif.) and the MAPK (erk-2) antibody was purchased from Upstate Biotechnology Incorporated (Lake Placid, N.Y.).

Assessment of cell death. (i) Trypan blue exclusion. Viability was assessed by light microscopic quantitation of trypan blue-excluding cells.

(ii) TUNEL assay. The ApopTag plus in situ apoptosis detection kit (Oncor, Inc., Gaithersburg, Md.) was used according to the manufacturer's instructions. Briefly, suspension cells were fixed in 4% neutral buffered formalin in phosphate-buffered saline (PBS), incubated 10 min at room temperature, collected by centrifugation, and resuspended in 80% ethanol. Cells were immobilized onto

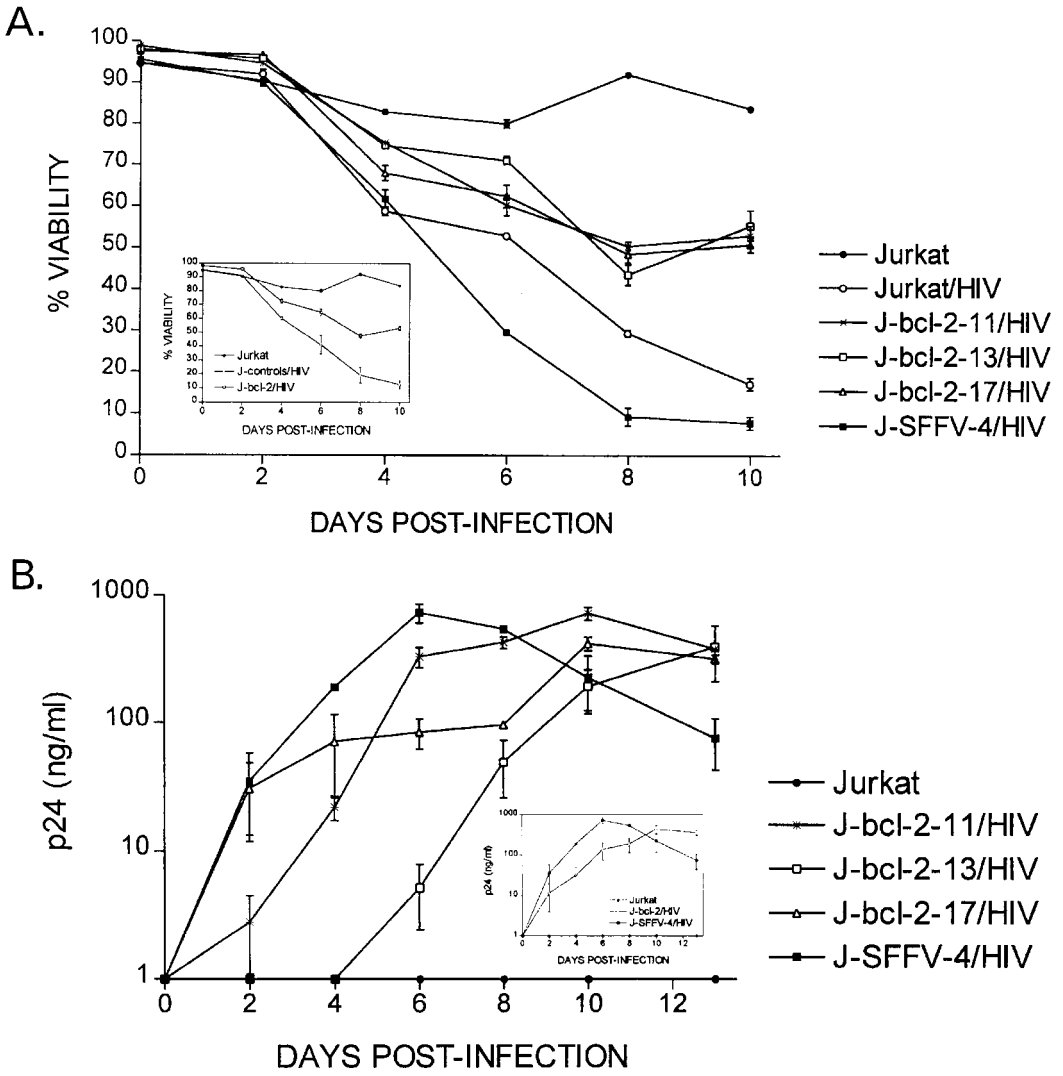

FIG. 1. Effect of Bcl-2 overexpression on HIV-1-induced cell death. Jurkat cells, Jurkat transfected with an empty vector (J-SFFV-4), and three Bcl-2-transfected cell lines (J-bcl-2-11, J-bcl-2-13, and J-bcl-2-17) were infected by HIV-1(LAI) as described in Materials and Methods. The results are presented as mean value ± the standard error of the mean of duplicate experiments. (A) Cell viability. Aliquots of cells were removed every other day and monitored for viability by trypan blue exclusion assay. (B) HIV-1 production was assayed by measuring the amount of p24 antigen by enzyme-linked immunosorbent assay. Inset, results of the average of the Bcl-2 cell lines versus the control cell lines infected with HIV-1. Data were analyzed by the paired *t* test, using a two-tailed *P* value. The *P* value was 0.0258 for the percent viability and 0.4792 for p24 production.

glass slides and washed twice with PBS. Detection of free 3′-OH ends was done by the addition of digoxigenin-nucleotide by terminal deoxynucleotidyl transferase (TdT) and revealed by the anti-digoxigenin-peroxidase antibody. Slides were counterstained in methyl green.

Cell cycle analysis. Cells were washed twice in Hanks' balanced salt solution (HBSS) and fixed in 70% ethanol for 3 h at 4°C. The cells were washed and resuspended in 0.5 ml of HBSS. Thereafter, 0.5 ml of RNase A (1 mg/ml of HBSS) and 1 ml of propidium iodide (PI) (100 μg/ml of HBSS) were added. After mixing, the cells were incubated overnight at 4°C in the dark. The PI fluorescence of individual cells was measured with a flow cytometer (FACScan; Becton-Dickinson, San Jose, Calif.).

Assay of PARP and D4-GDI cleavage. PARP and D4-GDI cleavage were detected on whole cell extract by immunoblotting, using a monoclonal anti-poly(ADP-ribose) polymerase antibody (Pharmingen, San Diego, Calif.) and an anti-D4-GDI rabbit polyclonal antibody (63), provided by G. M. Bokoch (Scripps Research Institute, La Jolla, Calif.). Briefly, the cells were pelleted, washed once in cold PBS, resuspended in sample buffer (125 mM Tris [pH 6.95], 15% sucrose, 4% SDS, 10 mM EDTA, 0.002% bromophenol blue, 100 mM dithiothreitol), and boiled for 5 min. The viscosity of the samples was reduced by several passages through a 23-gauge needle. Samples were boiled and run on 7.5% SDS-PAGE gels for PARP cleavage and on 15% SDS-PAGE gels for D4-GDI cleavage. The filters were blocked with 3% bovine serum albumin, incubated overnight with the primary antibodies and 1 h with the secondary antibodies, and developed with enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

Statistical analysis. Statistical analysis was done by the paired *t* test or the Mann-Whitney test, using a two-tailed *P* value and $P < 0.05$ as the criterion for statistical significance. These tests were used to compare two columns. When three or more groups were compared, the statistical method used was the one-way analysis of variance (one-way ANOVA). We have used the following: $P > 0.05$, not significant; *P* values between 0.01 to 0.05, significant; and $P \leq 0.01$, highly significant.

RESULTS

HIV infection results in cell death of Bcl-2 transfectants. Bcl-2 has been shown to protect T cells from apoptosis induced by diverse agents such as glucocorticoids, irradiation, anti-T-cell receptor antibodies, ionomycin, phorbol ester, sodium azide, heat shock and growth factor, or serum deprivation (11, 15, 61, 73). To study the effects of the Bcl-2 antiapoptotic protein on cell killing by HIV, we generated a series of cell

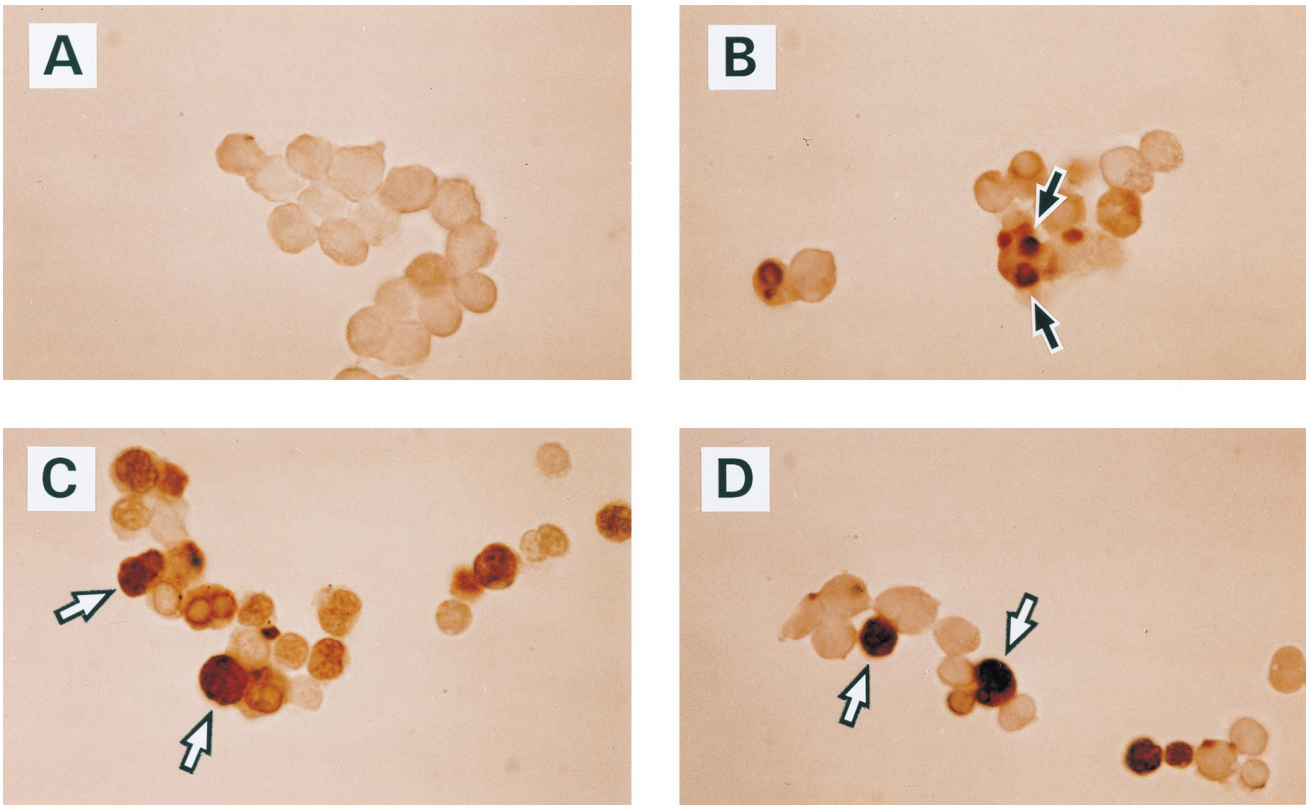

FIG. 2. Apoptosis analysis by TUNEL assay. Aliquots of cells were removed (day 8 postinfection) and fixed in 4% neutral buffered formalin, and 3′-OH ends were detected by the addition of TdT and revealed by the antidigoxigenin antibody peroxidase conjugate. (A) Jurkat cells. (B) Jurkat cells treated for 48 h with 5 mM NaB. Arrows, pyknotic nuclei, characteristic of cells undergoing apoptosis. (C) Jurkat cells infected with HIV-1(LAI). (D) Jurkat cell overexpressing Bcl-2 (clone J-bcl-2-11) and infected with HIV-1(LAI). Arrows in panels C and D, rimmed nuclear staining and open nuclei. Magnification, ×1,000.

lines transfected with either a control plasmid (SFFV-LTR) or with a plasmid directing the constitutive expression of the complete Bcl-2 protein (SFFV-Bcl-2-nl) under the regulation of the SFFV long terminal repeat (LTR) promoter and enhancer. Three independent Bcl-2 transfectants (J-bcl-2-11, J-bcl-2-13, and J-bcl-2-17) and two negative controls, the parental Jurkat cell line and a clone transfected with an empty vector (J-SFFV-4), were infected with the T-cell line-tropic, SI HIV-1(LAI) isolate. Among the Bcl-2 transfectants, cell viability measured as trypan blue-excluding cells was slightly (10 to 40%) but statistically significantly ($0.05 > P > 0.01$) higher than in control cells ($P = 0.025$) (Fig. 1A). For example, at day 10 postinfection, control cells were less than 20% viable, while Bcl-2 transfectants were between 45 to 50% viable. The difference in HIV replication between transfectants and controls was not statistically significant ($P > 0.05$) (Fig. 1B).

To confirm these results, we performed TdT-mediated DNA end labeling (TUNEL assay) of HIV-infected cultures to detect nicked DNA, characteristic of dying cells. TUNEL staining cells were absent from control cultures (uninfected Jurkat cells) (Fig. 2A), consistent with their high viability. In contrast, numerous TUNEL-staining cells were detected in Jurkat (Fig. 2C) and Bcl-2 (Fig. 2D) transfectants after HIV infection. These TUNEL-positive cells did not form characteristic nuclear apoptotic bodies (Fig. 2B), and DNA isolated from these cultures was smeared instead of displaying an apoptotic ladder (data not shown). Most of the TUNEL-positive cells in HIV infection had a single nucleus (Fig. 2), which we interpreted to be consistent with previous reports that HIV-1 isolates induce mainly single cell killing (62).

Bcl-2 protects Jurkat cells from NaB induced apoptosis. To characterize the functional properties of transfected Bcl-2 in Jurkat cells, cultures were treated with 5 mM sodium butyrate (NaB) for 48 h to induce apoptosis (57) and monitored by flow cytometry for DNA content and by TUNEL assay for damaged DNA. NaB-treated Jurkat cells showed a distinct apoptotic peak (60.8%) of cells with a DNA content below G_0/G_1 (Fig. 3A), which was greatly reduced (to 9.0%; 85% inhibited) in Bcl-2 transfectants. Bcl-2 expression did not relieve NaB-induced G_1 phase cell cycle arrest, as indicated by fewer S phase and G_2 phase cells in both Jurkat (from 10.7 to 2.8%; 74% reduction) and Jurkat-(bcl-2) (from 15.1 to 5.0%; 67% reduction) cultures after NaB treatment (Fig. 3A). When cells were examined by TUNEL assay for the presence of apoptotic bodies, many of the NaB-treated Jurkat cells had brown-staining apoptotic nuclei, which were not observed in the Bcl-2-expressing transfectants (Fig. 3B). These results together confirmed that transfected Bcl-2 functioned as an inhibitor of apoptosis in Jurkat T cells and that cell cycle arrest could be induced independently from apoptosis in T cells as previously reported (45).

Immunoblots were performed on protein lysates from NaB-treated cells (Fig. 4) and analyzed for cyclin B and phosphorylated cdc2 protein characteristic of T cells in S or G_2 phases. Consistent with its induction of cell cycle arrest and apoptosis of cells from G_1 phase, NaB treatment dramatically reduced cyclin B and phosphorylated cdc2 proteins in Jurkat cells (Fig. 4, lanes 2 and 6). G_1-arrested, NaB-treated Bcl-2 transfectants also had decreased levels of cyclin B and phosphorylated cdc2 proteins (Fig. 4, lanes 4 and 8). These did not appear to be nonspecific changes, because the amount of ERK-2 kinase in the cells

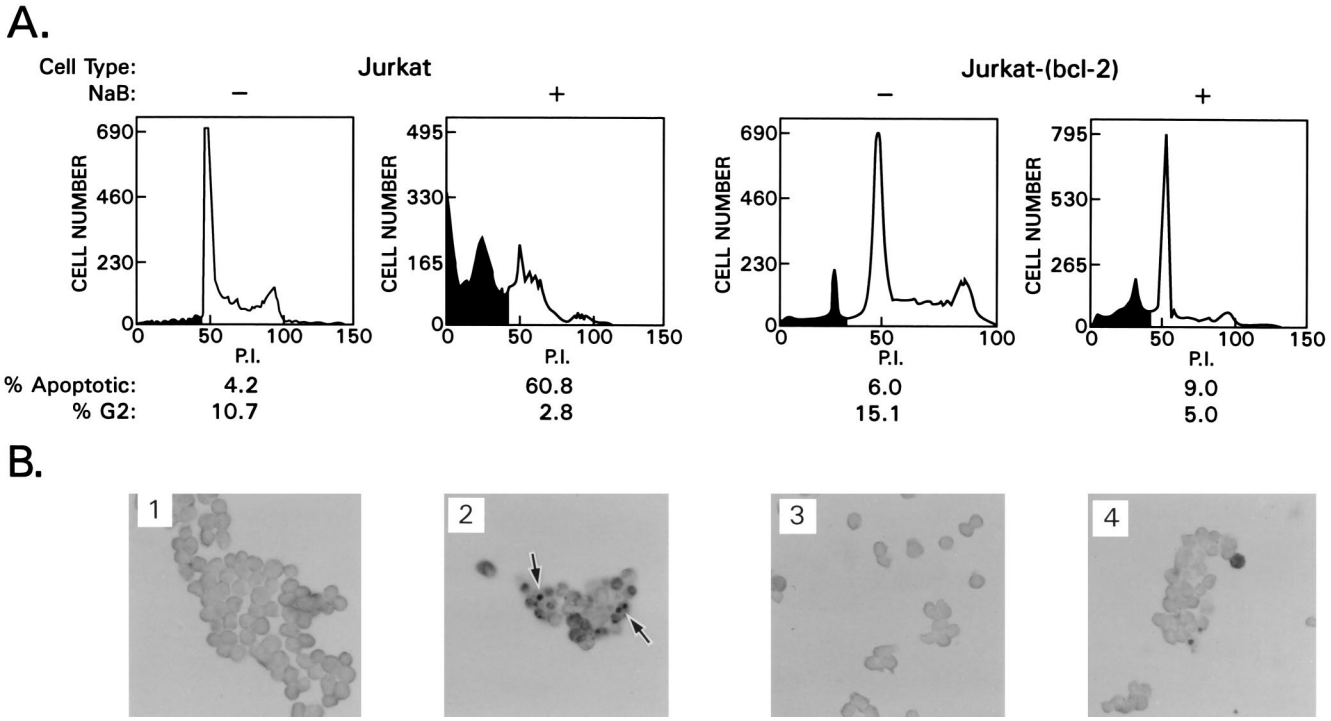

FIG. 3. Characterization of functional Bcl-2 and detection of apoptosis after treatment with NaB. (A) Jurkat cells were treated with NaB (5 mM) for 48 h, stained with PI, and analyzed by flow cytometry. Apoptotic nuclei appeared as a distinct hypodiploid DNA peak. (B) Cells were stained by the TUNEL technique: 1, Jurkat cells; 2, Jurkat cells treated with NaB; 3, J-bcl-2-11; 4, J-bcl-2-11 treated with NaB. Arrows, apoptotic bodies. Magnification, ×40.

was not altered by NaB treatment (Fig. 4). This ERK-2 antibody detects total protein but does not analyze its specific activity. These findings regarding apoptosis in Jurkat cells can be compared to our previous findings regarding HIV envelope-induced Jurkat T-cell death, in which increases in cyclin B and phosphorylated cdc2 proteins indicate that cells are accumulating in cell cycle S and G_2 phases during this form of cell death (39).

HIV-1-initiated cell killing in E1B 19K transfectants. E1B 19K protein has a broader antiapoptotic spectrum than Bcl-2, and it has been suggested to be more effective than Bcl-2 in inhibiting apoptosis by FasR (26) or TNFR (77). To study the effect of E1B 19K protein in HIV infection, we infected Jurkat cells overexpressing the E1B 19K protein (3) with HIV-1 (LAI). Between 45 to 50% of the E1B-expressing cells remained viable at day 10 postinfection (Fig. 5A), compared to 15% viability in the control infections ($P = 0.017$). E1B 19K antiapoptotic activity in our cell lines was assessed by treatment with NaB. More than 90% of the E1B 19K transfectants were still viable when treated with NaB, compared to only 20% in the control cells. HIV propagation was monitored by p24 assay, which showed that viral replication was more sustained between days 6 and 13 for the E1B 19K transfectants than for the control ($P = 0.015$) (Fig. 5B, inset). The peak amount of

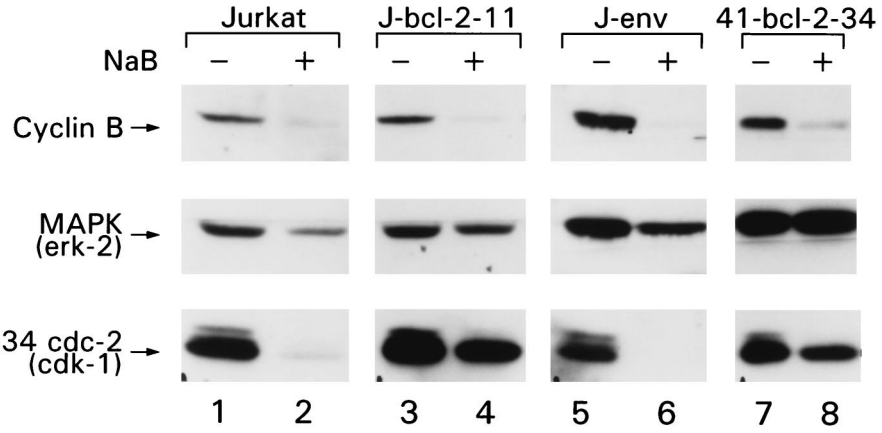

FIG. 4. Cyclin B/cdc2 disregulation is not a component of NaB-induced apoptosis. Cells were stimulated for 48 h with NaB (5 mM), and protein extracts were resolved on an 11% polyacrylamide gel. The immunoblots were probed for cdc2 (cdk1) with a polyclonal anti-cdc2, for MAPK (erk-2), and for cyclin B levels with a rabbit polyclonal anti-cyclin B antibody. Lanes 2, 4, 6, and 8, cells treated with NaB; lanes 1, 3, 5, and 7, untreated cells.

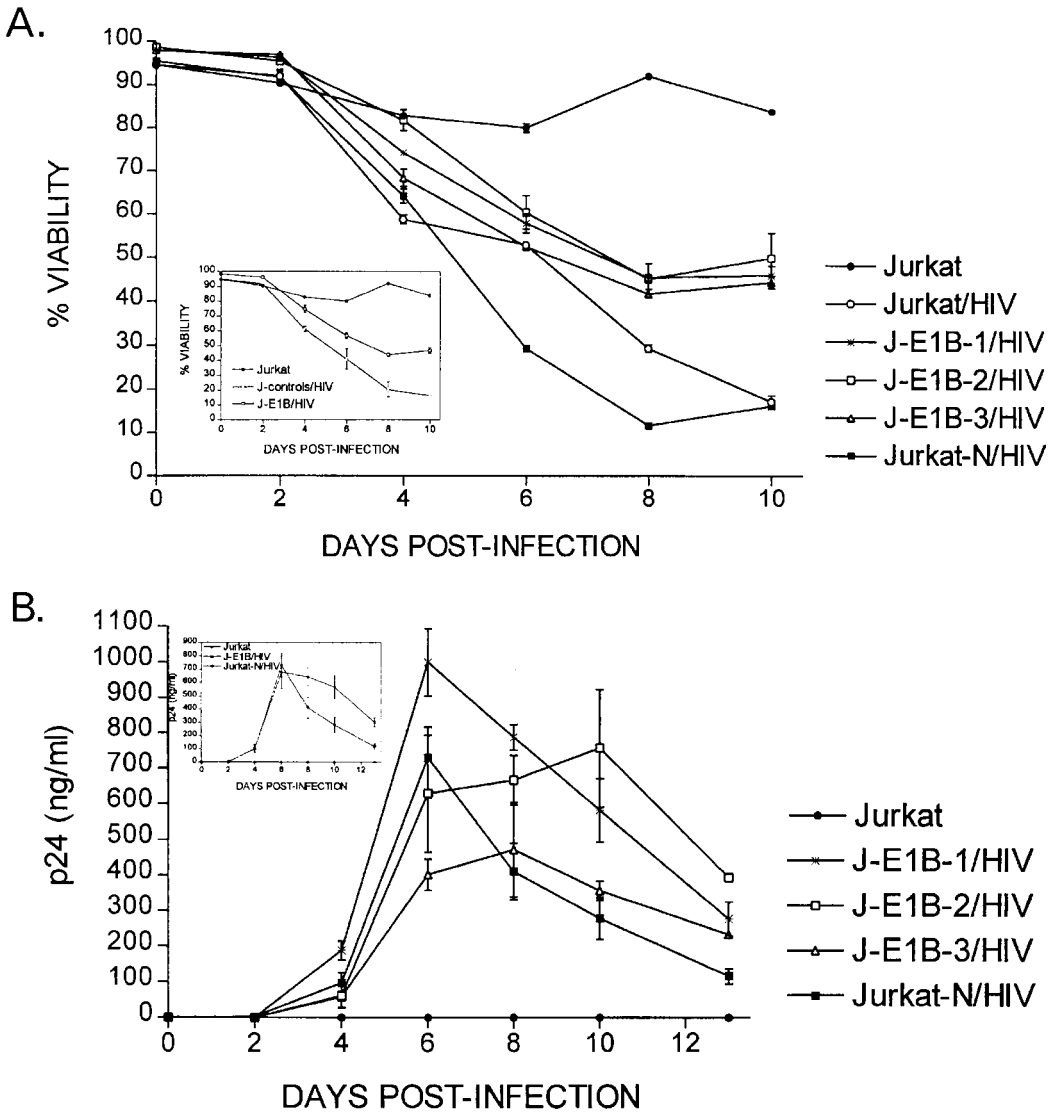

FIG. 5. HIV-1 replication and cell viability in Jurkat cells overexpressing E1B 19K protein. Three E1B 19K clones (J-E1B-1, J-E1B-2, and J-E1B-3) and one control cell line (Jurkat-N) were infected with HIV-1(LAI). Data are presented as the mean value ± standard error of the mean of duplicate experiments. (A) Cell viability. Aliquots of cells were removed, and cell viability was monitored by trypan blue exclusion. The data are the percentage of viable cells in the cultures. For each cell line a control was included (mock infection). (B) HIV-1 p24 antigen production was assessed by enzyme-linked immunosorbent assay. Inset, results corresponding to the average of E1B cell lines relative to control cell lines. Data were analyzed by the paired *t* test, using a two-tailed *P* value. The *P* value was 0.0174 for the percent viability, and 0.0152 for p24 production (*P* value, using data at days 8, 10, and 13 postinfection for p24).

viral replication was similar for both controls and E1B transfectants (approximately 700 ng/ml) (Fig. 5B).

Limited contribution of Fas-mediated apoptosis to T-cell death during in vitro HIV-1 infection. Although the preceding results from HIV infections of E1B and Bcl-2 transfectants supported an HIV-induced cell death pathway independent from FasR (APO-1/CD95), they also suggested a FasR-associated component. To study this possibility, HIV infections were performed in the presence of soluble immunoglobulin G (IgG)-Fas, to block Fas-initiated cell death, or in MOLT-4 T cells, which lack a critical intracellular phosphatase, which we confirmed renders them insensitive to Fas-mediated apoptosis (data not shown) (67). A small (5 to 10%) but statistically significant percentage of cell death ($P = 0.02$) was inhibited in HIV(LAI)-infected Jurkat cultures containing soluble IgG-Fas (10 μg/ml) when compared to control infections (Fig. 6A). MOLT-4 cells infected with HIV-1(LAI) had more cell death than Jurkat cells (Fig. 6; 70% versus 56% at day 8 postinfection), consistent with the higher levels of surface CD4 on MOLT-4 cells (data not shown) but inconsistent with Fas triggering the major component of acute T-cell death during these HIV infections.

To begin to define specific HIV genes contributing to Fas-initiated cell death, we studied previously described, stable HIV *env* transfectants (J-env cells). In this system, death is initiated by coculture between cells expressing surface HIV Env (gp120/gp41) with other $CD4^+$ T cells (68). Cell death was evaluated in the presence of anti-human Fas (ZB4) antibody to block Fas-mediated apoptosis (83) or in the presence of soluble IgG-CD4 to block HIV Env-CD4 interactions. Fifty percent of the cells died during the 48 h of J-env coculture, which was not influenced by the presence of anti-Fas antibody, ZB4,

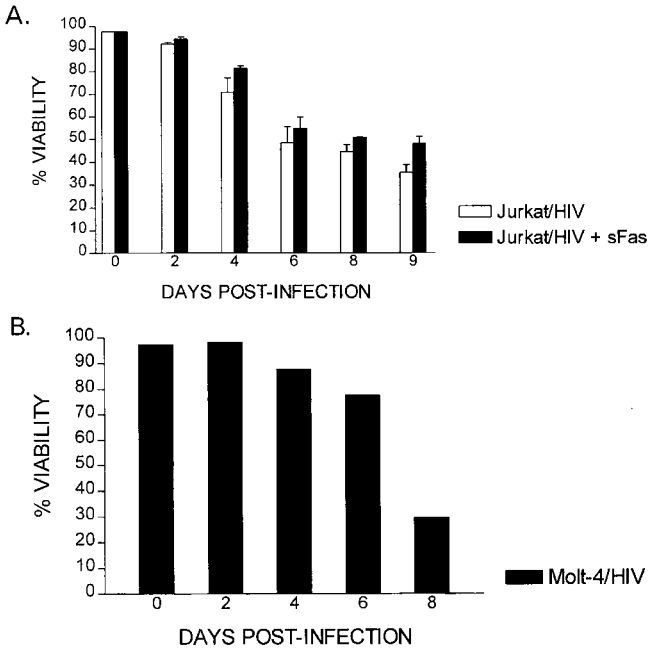

FIG. 6. Partial contribution of Fas-mediated apoptosis to T-cell death during in vitro HIV-1 infection. (A) Jurkat cells were infected with HIV-1(LAI) in the presence or absence of soluble IgG-Fas (10 μg/ml). Cell viability was assessed by trypan blue exclusion. Results are presented as mean ± standard error of the mean of duplicate experiments. Data were analyzed by the paired *t* test, using a two-tailed *P* value ($P = 0.0232$). (B) HIV-1 cell killing proceeds, despite a Fas-defective pathway in MOLT-4 cells. MOLT-4 cells were infected with HIV-1 (LAI). Aliquots of cells were removed every other day and measured by trypan blue exclusion for cell viability. The data are representative of four separate determinations.

in the cultures (Fig. 7A) ($P > 0.05$). As expected from its ability to inhibit the HIV Env-CD4 interaction, the addition of soluble IgG-CD4 to the cocultures completely blocked cell killing. Similar to our results with ZB4, Bcl-2 expression did not alter cell killing in this coculture system (Fig. 7B).

HIV Tat protein enhances Fas-triggered cell death that could be inhibited by Bcl-2 protein. Following reports from other laboratories that HIV Tat protein could enhance Fas-triggered apoptosis (42, 76) and because our J-env cell lines also expressed the Tat protein, we studied whether these cell lines might require an initiating signal to reveal their sensitivity to Fas-induced apoptosis. When Jurkat cell lines expressing Tat and HIV envelope (also expressing Rev) were stimulated to undergo apoptosis with the Fas antibody CH-11 (50 ng/ml) in a time course experiment (Fig. 8B), they were more susceptible to death at all time points (75 to 80% cell death at 3 h) compared to Jurkat cells (55% cell death at 3 h). Under these conditions, Bcl-2 expression (Fig. 8B, 41-bcl-2) rescued the cells from Fas-induced apoptosis in a strong and highly statistically significant manner ($P < 0.0001$). This increased susceptibility to Fas-mediated apoptosis was not due to alterations in Fas expression, which was equivalent in all of the lines (data not shown), did not require coculture with another $CD4^+$ cell, and was not inhibitable by soluble CD4. Bcl-2 expression had little effect on Fas-mediated apoptosis in the absence of Tat coexpression (Fig. 8A) ($P > 0.05$).

To examine the suggestion that HIV Tat protein was primarily responsible for enhancing the Fas-dependent component of HIV-mediated cell death, we transfected Jurkat cell lines with *tat*. Cell lines (J-tat-4, J-tat-5, and J-tat-6) were screened for *tat* RNA expression and for the ability to transactivate the HIV LTR. Both LTR-CAT and RSV-β-galactosidase constructs were introduced into the cell lines by lipofectin transfection, and chloramphenicol acetyltransferase (CAT) activity was normalized for β-galactosidase activity. Transient transfections were also done in Jurkat cells with a control plasmid (pCAT), and the CAT activity was 642 cpm (control). CAT activity for J-tat-5 (104,847) was comparable with the CAT activity detected for J-env (136,700 cpm) and J-env-overexpressing Bcl-2 (140,800 cpm). The CAT activities for the cell lines J-tat-4 and J-tat-6 were somewhat lower (20,801 and 39,086 cpm, respectively). Nevertheless, when any of these Tat-expressing cell lines were stimulated to undergo apoptosis with anti-human Fas antibody (CH-11) they were three- to fourfold more susceptible to Fas-induced apoptosis than controls (Fig. 8B). The range of cell death after anti-Fas treatment was similar to that previously observed in J-env cells (Fig. 8B).

PARP and D4-GDI cleavage in cell death induced by Fas but not in acute HIV infection. Activation of a cascade of ICE-related proteases, resulting in cleavage of the ICE substrate PARP and members of the pro-ICE family, may be a universal feature of apoptosis conserved in species from *C. elegans* to humans (36). To further characterize the apparently distinct Fas-induced and HIV *env*-induced cell death pathways, we studied cleavage of two ICE-family protease substrates, PARP and D4-GDI (63). FasR activation with anti-Fas antibody resulted in proteolytic cleavage of PARP to the expected 85-kDa fragment (Fig. 9A, lane 9), and D4-GDI was also cleaved to its 23-kDa apoptotic fragment (Fig. 9B lane 1). No PARP (Fig. 9A) or D4-GDI cleavage (Fig. 9B) accompanied HIV infection of Jurkat or SupT-1 cells, even though by day 6, respectively, 70 and 38% of the infected cells were dead. Moreover, infection of PBMC with an SI isolate [HIV-1(571)] (Fig. 9C) did not result in an increase of D4-GDI cleavage above background, despite extensive cell death (39.5% cell mortality at day 8 post-infection), although we note that the background of cleaved D4-GDI is higher in these PBMC cultures (Fig. 9C). In addition, PARP was not cleaved during cell death induced by HIV envelope in the J-env coculture system (data not shown).

DISCUSSION

The precise mechanisms of cell killing during HIV infection have not been satisfactorily explained and are likely multifactorial, depending on the stage of disease progression. A series of strategies designed to block apoptosis in T cells could not inhibit most cell death occurring during HIV-1 infection. These studies were complemented by others establishing that ICE cleavage products were not readily detected during acute HIV infection despite extensive cell killing. Nevertheless, a component of cell death during acute HIV infection had features characteristic of apoptosis, including inhibition by the antiapoptotic proteins E1B and Bcl-2 and competition by anti-FasR antibodies. This pathway was not the major contributor to cell killing during acute HIV infection, likely explaining our inability to detect PARP cleavage despite the extensive cell death in these infections.

These data are most easily explained by the existence of at least two HIV stimuli for cell death. It has recently been shown that Fas-FasL interaction and/or the upregulation of Fas expression on the surfaces of cells from HIV-infected individuals can contribute to HIV-mediated cell death (4, 38). It was further demonstrated that Tat protein could enhance Fas-triggered apoptosis (42, 53, 76, 85) under most circumstances (43). In agreement with these results demonstrating apoptosis in HIV infection, when Tat-expressing cell lines were stimulated to undergo apoptosis with anti-human Fas antibody (CH-11),

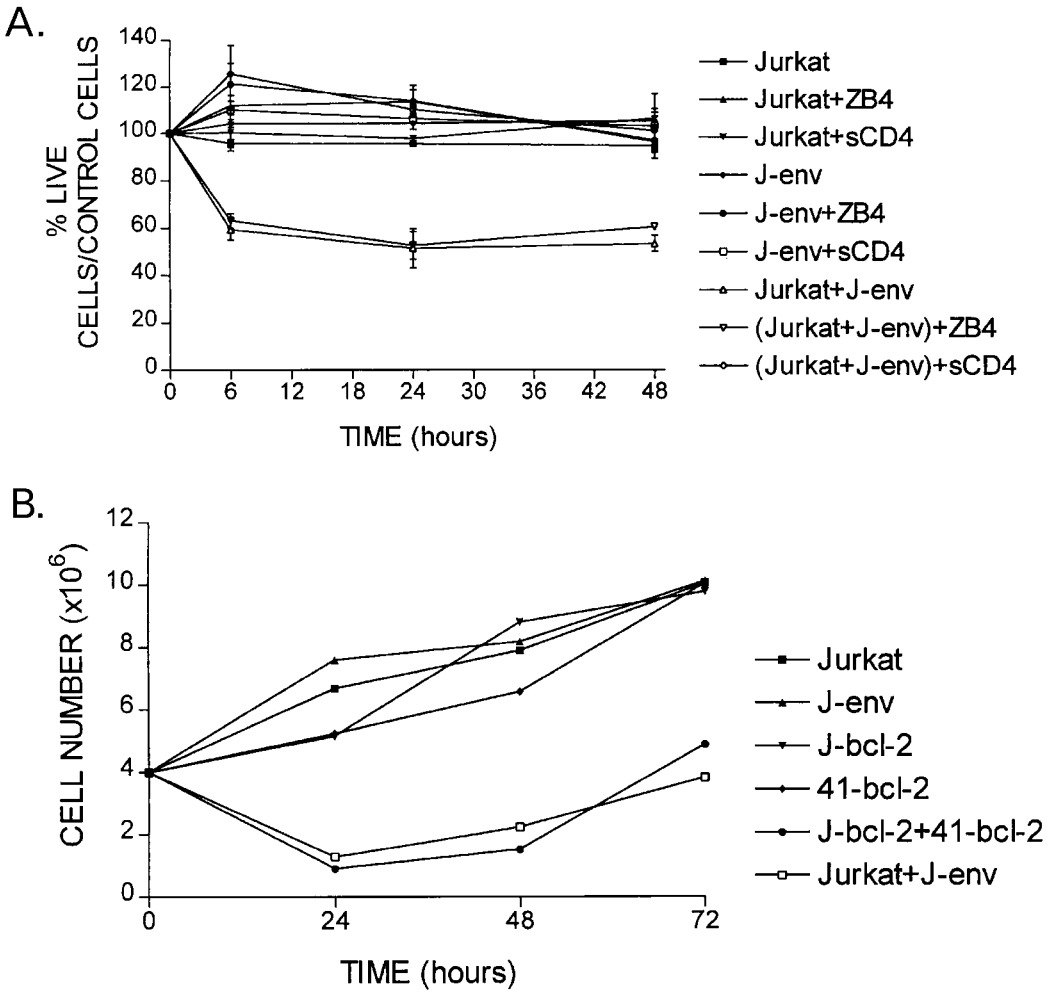

FIG. 7. J-env-mediated cell death. Jurkat T cells and T-cell transfectants stably expressing HIV envelope glycoproteins (J-env cells) were mixed in a 1/1 ratio to induce cell killing initiated by the interaction of the HIV envelope glycoproteins (gp120/gp41) with CD4. (A) Some cultures were done in the presence of soluble IgG-CD4 at a concentration of 10 μg/ml. To inhibit Fas-induced apoptosis, some cells were pretreated with the anti-human Fas antibody, ZB4, at a concentration of 250 ng/ml for 1 h and maintained in anti-ZB4 antibody (250 ng/ml) during cultures. Viability was monitored by trypan blue exclusion. For each time point the percentages of live cells to control cells was calculated as follows: (live cells) × 100/(mean of live Jurkat + J-env cells). Data were analyzed by one-way analysis of variance. Results are presented as mean value ± standard error of the mean of duplicate experiments. (B) CD4 cells transfected to overexpress Bcl-2 (J-bcl-2) were mixed in a 1/1 ratio with J-env cells also expressing Bcl-2 to induce HIV envelope-initiated cell death in the population. Cell viability was assessed by trypan blue exclusion. The data are representative of four separate determinations.

they were much more susceptible to apoptotic cell death than controls.

Even though Tat, and likely other HIV proteins, appeared to augment AICD during HIV disease, this apoptosis seemed to contribute only a lesser component to HIV-induced cell death. The major part of cell death during HIV infection instead proceeded by a nonapoptotic pathway. Supporting this conclusion, no evidence for ICE-related proteolysis during acute HIV infection was observed in this report. Activation of the ICE cysteine protease cascade plays an evolutionarily conserved and critical role in triggering apoptosis (36). In the case of T-cell apoptosis, FasR is most closely linked to the ICE protease, FLICE (7, 50). ICE proteases stimulated in the apoptotic pathway appear to invariantly target a specific set of endogenous substrates, including PARP and other pro-ICE family members. Whereas under specific experimental conditions ICE family proteolysis may be bypassed (80), all cellular pathways of apoptosis described to date trigger members of the ICE family. Since it might be expected that at least HIV Tat-associated apoptosis would stimulate PARP cleavage, it seems possible that this activity is too gradual and contributes too small a component to be detected in our biochemical analysis of acute cytolytic infections. This possibility preserves a role for apoptosis in the gradual decline and immune disregulation associated with the plateau phase of HIV infection and would be entirely consistent with a previous report suggesting that protease inhibitors could partially restore defective in vitro T-cell responses in HIV-positive donors (59).

Our results show that $CD4^+$ T cells transfected to express E1B 19K protein or Bcl-2 were not protected from HIV-initiated killing, although they became resistant to Tat-enhanced or NaB-mediated apoptosis. Sandstrom et al. (58) reported that Bcl-2 overexpression accelerated HIV-1 replication, probably by limiting syncytial apoptosis, but we and others (52) have not observed the same level of effect for Bcl-2. We found that E1B 19K protein expression blocked some HIV-induced cell death and was associated with a more sustained viral production, which is in partial agreement with the findings of

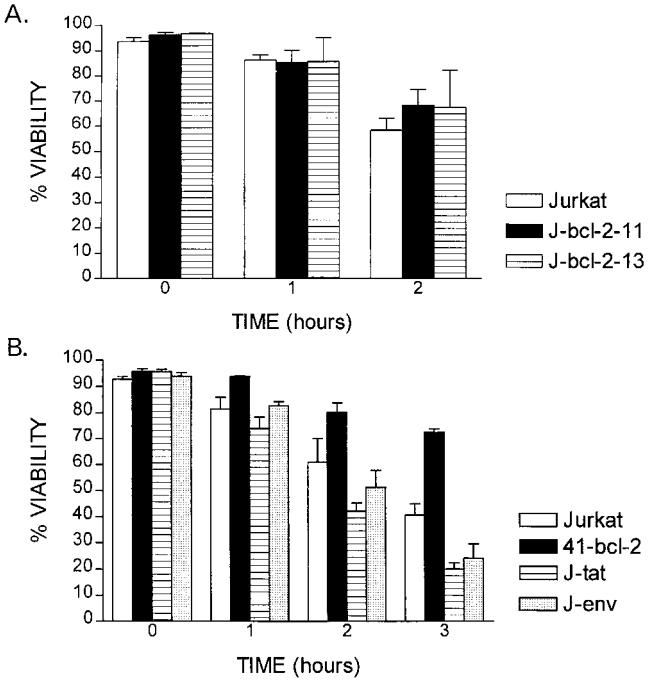

FIG. 8. Participation of FasR and Tat in HIV-triggered apoptosis. Cells were cultured in the presence of the anti-human Fas antibody, CH-11 (50 ng/ml), to induce cell death. At various times, aliquots of cells were removed and monitored for viability by trypan blue exclusion. (A) Percent viability of two Jurkat clones overexpressing Bcl-2 (J-bcl-2-11 and J-bcl-2-13) at 0, 1, and 2 h after treatment with Fas antibody CH-11. The results are presented as mean value ± standard error of the mean of duplicate (J-bcl-2-13) or triplicate (J-bcl-2-11 and Jurkat) experiments. The *P* value was not significant. (B) Bcl-2 protects cells from Fas-initiated apoptosis, and Tat expression accounts for HIV-1 enhancement of apoptosis. Jurkat HIV *env* cells overexpressing Bcl-2 (41-bcl-2) show resistence to apoptosis when stimulated with Fas antibody (CH-11). Tat-expressing cell lines (J-tat) and cell lines expressing the HIV envelope gene and also the *tat* gene (J-env) are more susceptible to Fas-induced apoptosis. Results are presented as mean value ± standard error of the mean of the average of three cell lines for 41-bcl-2 (clones 6, 11, and 34) and for J-tat (clones 4, 5, and 6) and of two cell lines for J-env (one expressing gp120 + gp41 and the other expressing gp160). Data were analyzed by the Mann-Whitney test, using a two-tailed *P* value.

Antoni et al. (3). Nevertheless, in our infection conditions, most of the HIV-induced death could not be blocked by E1B protein. This apparent contrast is most likely explained by the different cell lines and the different multiplicities of infection used by Sandstrom et al. and Antoni et al., both of which apparently favored syncytium formation, and syncytial apoptosis, as opposed to the single cell killing observed in our infections (Fig. 2). Single cell killing of T cells appears to predominate during infection of PBMC with primary cytopathic SI isolates of HIV-1 (12, 13).

The adenovirus E1B 19K product and Bcl-2 have structural and functional homology, but E1B 19K has been found more effective than Bcl-2 for inhibiting TNF- and Fas-induced cell death (our data not shown and references 26, 66, and 77). While we found that Bcl-2 protein strongly inhibited Fas-initiated PCD in the context of HIV Tat protein expression (Fig. 8), neither E1B nor Bcl-2 could strongly antagonize cell death during HIV infection. Fas independence of this HIV death pathway was further supported by infection of MOLT-4 T cells. These cells express FasR but are not susceptible to Fas killing (67). Despite a defective Fas signaling pathway, MOLT-4 cells are efficiently killed by HIV infection (reference 41 and our results).

Our studies on cell death of envelope transfectants (J-env cells) support the conclusion that the major nonapoptotic HIV cytopathicity is initiated by envelope glycoproteins (gp120/gp41). This possibility is consistent with studies mapping the cytopathic effect of HIV infection to the envelope glycoproteins. J-env-induced cell death was not blocked by Bcl-2 (Fig. 7B), nor did J-env-initiated cell death result in PARP cleavage. We previously demonstrated in T-cell lines that J-env-induced cell death is associated with aberrant cell cycle transition through the S and G_2 phases (39). In contrast, AICD in T cells is typically associated with cell cycle disregulation and cell death from the G_1 phase (45) (Fig. 3). The morphological changes accompanying HIV Env-induced cell death include balloon degeneration and cytoplasmic vacuolization of single cells (8, 39). Many of these features are more typical for necrosis than for apoptosis (8). These balloon changes are not characteristic of apoptotic cells, which have a shrunken cy-

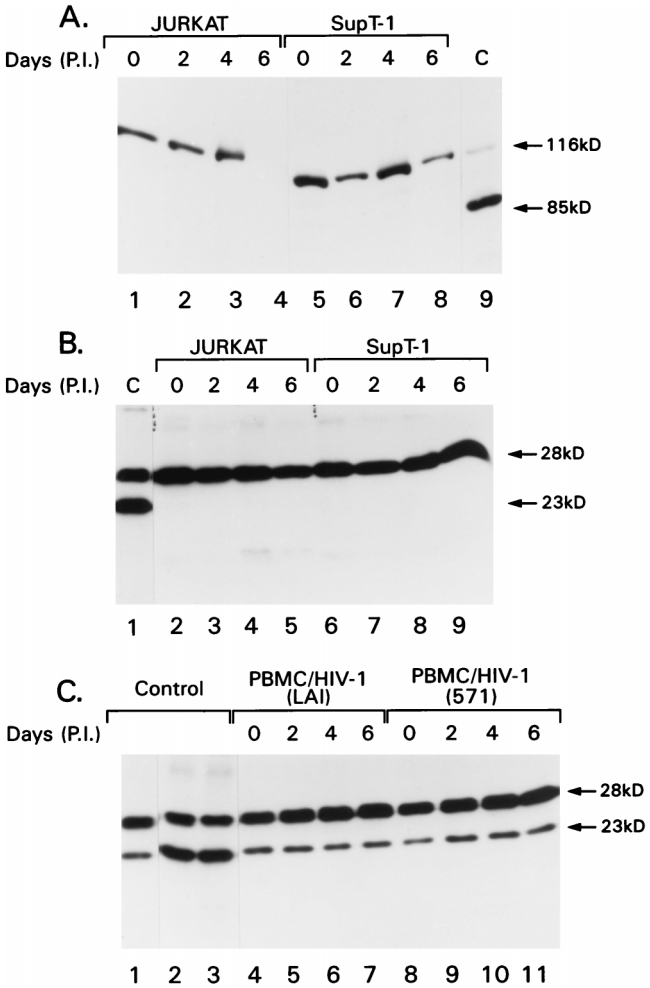

FIG. 9. No activation of ICE-like proteases in HIV-initiated cell death. (A) The cleavage of PARP was assessed by immunoblot analysis. Jurkat (lanes 1 to 4) and Sup T-1 (lanes 5 to 8) cells were infected with HIV-1(LAI). The positive control (lane 9), represents Jurkat cells treated with anti-Fas antibody (50 ng/ml) for 5 h. The 85-kDa fragment represents the apoptotic fragment of PARP. (B) Assay for D4-GDI cleavage. Cells were infected with HIV-1(LAI) and examined for D4-GDI cleavage. Only Jurkat cells treated with anti-Fas antibody (control, lane 1) show the expected 23-kDa apoptotic fragment. (C) PBMC were infected with HIV-1(LAI) (lanes 4 to 7) or with a clinical isolate [HIV-1(571)] (lanes 8 to 11) and were tested for D4-GDI cleavage. Lanes 1, 2, and 3 represent PBMC treated with NaB for 0, 24, and 48 h, respectively.

toplasm and a pyknotic nucleus (apoptotic bodies). When TUNEL labeling was performed to detect dying cells, T cells undergoing apoptosis had characteristic apoptotic bodies (Fig. 1B), but TUNEL-staining cells observed during HIV infection typically did not (Fig. 1C and D).

Other HIV proteins in addition to Tat also appear to be capable of inducing apoptosis (65). Stimulation of CD4-mediated AICD by soluble, recombinant external HIV envelope glycoprotein has been extensively studied (6, 40, 41, 49, 69, 76). The interaction of gp120 with CD4 has been reported to activate PCD and intracellular signalling, including Ca^{2+} influx (48) and the CD4-associated protein kinase Lck (23, 31). Recently, other studies have shown that the HIV Vpr product can induce apoptosis (64) as well as cell cycle arrest at G_2 phase (27, 37, 55). While it appears likely, based on its predominance in T-cell PCD, that FasR/TNFR triggering would participate in any apoptosis induced by these HIV proteins, and therefore would be inhibitable by the strategies employed in this report, formal proof for this postulate is still lacking in most cases.

In conclusion, our data demonstrate that a major component of HIV-initiated cell death, likely contributed by the HIV envelope because it is seen in HIV envelope-expressing transfectants, proceeds by a Fas-independent pathway. Our studies further suggest that Fas-induced apoptosis in Jurkat cells is enhanced by HIV Tat expression and that this apoptosis could be limited by the E1B 19K or the Bcl-2 protein. Competition with apoptosis accelerates the replication of viruses as diverse as adenovirus and HIV-1, making PCD a relatively unattractive target for antiviral therapeutics. It remains to be definitively established whether the nonapoptotic pathway of cell death described here inhibits or promotes viral spread, in which latter case its inhibition might lead to a reduction in both cell death and in viral production. This latter possibility is suggested from studies linking HIV-related cytopathicity with high levels of viral replication (32, 75).

ACKNOWLEDGMENTS

We thank Maria Holland and Gretta Blatner for technical assistance, Eileen White for the E1B 19K cell lines, Pierre Henkart and Genoveffa Franchini for critical reviews of the manuscript, G. M. Bokoch for the D4-GDI antibody, and M. Reitz, Jr., for HIV-1 (G571) virus.

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