The HIV-1 vpr Protein Acts as a Negative Regulator of Apoptosis in a Human Lymphoblastoid T Cell Line: Possible Implications for the Pathogenesis of AIDS

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Summary

Although apoptosis is considered one of the major mechanisms of $CD4^+$ T cell depletion in HIV-infected patients, the virus-infected cells somehow appear to be protected from apoptosis, which generally occurs in bystander cells. Vpr is an auxiliary HIV-1 protein, which, unlike the other regulatory gene products, is present at high copy number in virus particles. We established stable transfectants of $CD4^+$ T Jurkat cells constitutively expressing low levels of vpr. These clones exhibited cell cycle characteristics similar to those of control-transfected cells. Treatment of control clones with apoptotic stimuli (i.e., cycloheximide/tumor necrosis factor α (TNF-α), anti-Fas antibody, or serum starvation) resulted in a massive cell death by apoptosis. In contrast, all the vpr-expressing clones showed an impressive protection from apoptosis independently of the inducer. Notably, vpr antisense phosphorothioate oligodeoxynucleotides render vpr-expressing cells as susceptible to apoptosis induced by cycloheximide and TNF-α as the control clones. Moreover, the constitutive expression of HIV-1 vpr resulted in the upregulation of bcl-2, an oncogene endowed with antiapoptotic activities, and in the downmodulation of bax, a proapoptotic factor of the bcl-2 family. Altogether, these results suggest that low levels of the endogenous vpr protein can interfere with the physiological turnover of T lymphocytes at early stages of virus infection, thus facilitating HIV persistence and, subsequently, viral spread. This might explain why apoptosis mostly occurs in bystander uninfected cells in AIDS patients.

The human immunodeficiency virus type I (HIV-1) displays a high level of genetic complexity, which accounts for its tightly regulated replication. In addition to the structural and replicative proteins (gag, pol, and env), HIV-1 genome specifies at least six auxiliary proteins (vif, vpr, tat, rev, vpu, and nef) that are capable of regulating viral replication and infectivity (1, 2). The vpr accessory gene encodes a small basic protein (15 kD) that, unlike the other regulatory gene products, is present at high copy number in viral particles (3–5). Incorporation of vpr into HIV-1 virions is mediated by a specific interaction with the COOH-terminal region of the gag precursor (6–8). Because of its virion association, it has been suggested that vpr has an early role in HIV-1 infection, thus facilitating the transport of the virus core into the nucleus of nondividing cells. Subsequently, it has been reported that, together with the viral matrix (MA) protein, vpr plays a fundamental role in the proviral DNA integration process by connecting the preintegration complex with the cell nuclear import pathway (9, 10).

The importance of vpr for viral persistence, replication, and pathogenesis is suggested by a number of in vivo and in vitro studies. In particular, it has been demonstrated that, in macaques infected with wild-type or vpr-mutant viruses, vpr is associated with an increased viral load and rate of progression to AIDS (11). Moreover, it has also been shown that the vpr-positive strains grow faster and produce moderately higher levels of virus than their vpr-negative counterparts. This enhanced virus production is more pronounced in primary macrophages, suggesting that vpr function may be important in specific target cells (12–14). Interestingly, this protein does not appear to confer a significant viral growth advantage in primary T cells (15, 16). A few reports have also described effects of vpr on cell cycle and differentiation. In fact, HIV-1 vpr expression was first noted to promote differentiation and growth inhibition of a human rhabdomyosarcoma cell line (17). Subsequent studies revealed that vpr produces an accumulation of cells in the G_2/M phase of the cell cycle, thereby preventing the establishment of chronic HIV-1 infection in T lymphocytes (18–

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22). In some of these studies, vpr was shown to interact with upstream regulators of the cyclin-associated p34cdc2 kinase, which regulates the G_2/M transition (20, 21).

Apoptosis is a regulated mechanism of cell suicide that is essential for normal development and homeostasis in multicellular organisms and provides a defense against virus invasion and oncogenesis (23). Recent evidence suggests that most eukariotic cells respond to viral disruption of cellular homeostasis by undergoing apoptosis (24). To counteract this, many viruses have evolved mechanisms to block host cell death. In several cases, viral genomes have been found to possess genes whose products are capable of modulating, either positively or negatively, apoptosis of their host cells (25). Among the known examples of viral gene products blocking apoptosis are the adenovirus E1B protein (26), vaccinia CrmA protein (27), simian virus 40 T antigen (28), human papilloma 16 E6 protein (29), insect baculovirus p35 and iap proteins (30, 31), Epstein-Barr virus BHRF1 protein (32), human cytomegalovirus IE1 and IE2 gene products (33), herpes simplex virus 1 ICP4 (34) protein, and the very recently described bcl-2 homologue of human herpes virus 8 (35). As regards to HIV, some apparently contrasting data are available on the possible role of the HIV-1 Tat protein in the control of apoptosis. In particular, while Zauli and coworkers (36, 37) showed that Tat-expressing cells were resistant to different apoptotic stimuli, including acute HIV infection, other groups (38–41) have found that Tat induces apoptosis in T cells. Very recently, McCloskey et al. (42) demonstrated that Tat exhibited a dual role in the regulation of apoptosis in uninfected T cells. Although addition of exogenous Tat protein induced apoptosis in these cells, T cell clones stably expressing the Tat protein were protected from activation-induced apoptosis (42). Contrasting results have also been recently obtained on the effect of the HIV-1 vpr protein in the regulation of apoptosis (43, 44). In particular, Stewart et al. (43) have reported that the ability of vpr to arrest cells in the G_2 phase finally resulted in cell death by apoptosis. Furthermore, a recent study by Ayyavoo et al. (44) showed that vpr was capable of regulating, both positively or negatively, the T cell receptor triggered apoptosis depending on the state of immune activation.

In this study, we report that the constitutive expression of the HIV-1 vpr protein results in a marked inhibition of apoptosis induced by different stimuli, such as a combination of cycloheximide (CHX)[1] and TNF-α, anti-Fas antibody, and serum starvation. To the best of our knowledge, this is the first report in which a stable expression of low levels of the vpr protein has been achieved. The antiapoptotic effect of vpr is mediated, at least in part, by the regulation of the expression of some bcl-2 family members. These data suggest that the HIV-1 vpr protein is an early regulator of apoptosis in HIV-infected T cells and represents an additional survival strategy for HIV persistence and spreading.

[1] *Abbreviations used in this paper:* CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; PI, propidium iodide.

Materials and Methods

Construction of vpr Expression Plasmids

The vpr open reading frame of the HIV-1 genomic clone pBT-1, BRU strain (American Type Culture Collection, Rockville, MD) was amplified by PCR with oligonucleotides 5′CCCAAGCTTGCCGCCACCATGGAACAAGCCCC and 5′GCTCTAGACTAGGATCTACTGGCTCC. Products were digested with XbaI and cloned into the XbaI restriction site of the pRc/CMV plasmid (Invitrogen, Carlsbad, CA), whose promoter consists of a fragment of DNA from the immediate early gene of human CMV. Sequences from the bovine growth hormone gene are 3′ to the vpr gene to provide both polyadenylation and transcription termination site. The sequence of the vpr gene in the expression construct was determined before transfection and proved to be identical to the sequence of the molecular clone used as template for vpr amplification (data not shown).

Cell Culture and Production of vpr Transfectants

The Jurkat human T cell line (clone E6-1; obtained from National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD) was maintained in RPMI 1640 with 10% FCS and penicillin/streptomycin sulfate in 5% CO_2 atmosphere. Vpr-expressing cells were isolated after electroporation (500 μF, 250 V) of 5×10^6 cells with 10 μg of either pRc/vpr or pRc/neo plasmid DNA. The transfected cells were grown in RPMI containing 10% of FCS for 48 h before the addition of G-418 (1 mg/ml). After 4 d of growth in selection medium, the neomycin-resistant cells were seeded in RPMI containing 1% methylcellulose and 1 mg/ml G-418 in order to isolate single vpr-positive cell clones. The clones were maintained in RPMI medium containing 500 μg/ml of G-418 and analyzed for vpr expression.

Expression of vpr in Transfectants

The expression of vpr messenger RNA (mRNA) in neomycin-resistant clones was analyzed by RNA-PCR. For the reverse transcription, 1 μg of total RNA, extracted from cells harvested during the logarithmic growth by the method of Chirwgin (45), was mixed with 1 μg of oligo dT (12–18 oligomer; Pharmacia Biotech, Piscataway, NJ) as previously described (46). The cDNA obtained was amplified by using 0.1 μg of primers specific for the vpr or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a reaction mixture containing 200 μM dNTP and 0.5 U of Thermus acquaticus (TAQ) DNA polymerase in PCR buffer (44). PCR amplification was performed in a thermal controller (MJ Research, Inc., Watertown, MA) for 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. A negative control lacking template RNA or reverse transcriptase was included in each experiment (data not shown). The reaction products were visualized by electrophoresis on 2.5% agarose gels followed by ethidium bromide staining. 0.5 μg of HaeIII digested ϕX174 DNA (BioLabs, Inc., Beverly, MA) was used as molecular weight marker. The sequences of the GAPDH primers have been previously described (46).

For the detection of vpr protein, the neomycin-resistant cells were lysed in a buffer containing 1% Brij 96 as previously described (10). The cell lysates were precleared for 1 h at 4°C with protein G–Sepharose (Pharmacia Biotech) and then incubated overnight at 4°C with protein G–Sepharose precoated with a guinea pig anti-vpr antibody (10). After extensive washes with the lysis buffer, immune complexes were eluted, subjected to 18% SDS-PAGE, and transferred to filters (ClearBlot, ATTO). The filters were then processed for Western blotting with a guinea pig anti-vpr antibody (10).

Inhibition of vpr Expression by Antisense Phosphorothioate Oligodeoxynucleotides Targeted to vpr

Vpr-expressing clones were treated with phosphorothioate oligodeoxynucleotides targeted at the open reading frame of vpr as previously described (13). In brief, the cells were seeded at the concentration of 5×10^5/ml in the presence or in the absence of 20 μM of either antisense (oligonucleotide 3) or control sense (oligonucleotide 4) phosphorothioate oligonucleotides (13). After 18 h at 37°C, both oligonucleotides were added again and 1 h later cells were stimulated to undergo apoptosis. The extent of apoptosis was then evaluated after 4 h by propidium iodide staining.

Analytical Cytology

Cell Cycle Analysis. DNA analysis was performed by using propidium iodide (PI; 40 μg/ml) as previously described (47). At least 10,000 events have been acquired (Lysys II Software; Becton Dickinson, Mountain View, CA). The percentage of cells in the different phases of the cell cycle were obtained by CellFIT software analysis. The median values were estimated by three different methods: RFIT, SFIT, and SORB.

Apoptosis Assays. To evaluate apoptosis, both immunofluorescence and flow cytometry techniques were used. The chromatin dye Hoechst 33258 (Molecular Probes Inc., Eugene, OR) was used to visualize chromatin condensation and clumping as described elsewhere (47). Cells were fixed with paraformaldehyde 3% in PBS. After washing, all the samples were mounted with glycerol/PBS (1:1) and observed with a Nikon Microphot fluorescence microscope. Quantitative evaluation of apoptotic cells was performed by counting at least 12 microscopic fields in quadruplicate for a total amount of at least 400 cells at high magnification (×500). For DNA content evaluation by flow cytometry, the cells were fixed and permeabilized as previously described (48). The samples were then analyzed on a FACScan® flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Data were recovered by a Hewlett Packard computer using the Lysys II Software.

Quantitative Evaluation of Bcl-2 and Bax Proteins

Control and vpr-transfected Jurkat cells were pelleted, fixed in 70% ice-cold methanol, and washed twice with cold PBS. For Bax detection, cells were stained for 30 min at 37°C with an anti-bax polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, cells were incubated for 30 min at 37°C with FITC-labeled anti–rabbit polyclonal antibody (Sigma Chemical Co., St. Louis, MO). For the detection of bcl-2, cells were stained with an FITC-labeled anti–bcl-2 monoclonal antibody (Dako Corp., Carpinteria, CA) at a final concentration of 0.1 mg/ml for 30 min at 4°C. After washing, all samples were immediately analyzed on a FACScan® flow cytometer as described above. For Western blot analysis of bcl-2 and bax, whole cell extracts (60 μg), prepared as previously described (36), were resolved by 10% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and incubated with either anti–bcl-2 mouse monoclonal antibody (Dako Corp.; 1:100 dilution) or anti-bax rabbit polyclonal antibody (Santa Cruz Biotechnology; 1:100 dilution) overnight at room temperature. After four washes with TBS-T, membranes were reacted with peroxidase-conjugated anti–mouse (1:4,000 dilution) or anti–rabbit (1:1,000) immunoglobulin sera (Amersham Corp., Arlington Heights, IL) and visualized with the enhanced chemiluminescence detection system (Amersham Corp.). Membranes were further probed with antibody to β-tubulin (Sigma Chemical Co.; dilution 1:200) to ensure that an equal amount of proteins were loaded and transferred (data not shown).

Statistical Analysis

Statistical significance was evaluated by the Student's *t* test for correlated samples.

Reagents

CHX and human recombinant TNF-α were purchased from Sigma Chemical Co. Monoclonal IgM antibody to human Fas antigen was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). The anti-vpr antibodies were produced by immunizing guinea pigs with either recombinant HIV-1 vpr expressed by pGEMEX vector or a synthetic NH_2-terminal 14 oligopeptide of vpr (10).

Results

Establishment and Characterization of Jurkat Cell Clones Stably Expressing the HIV-1 vpr Protein. To obtain transfectants expressing the HIV-1 vpr gene, the open reading frame of the HIV-1 vpr, cloned into a pRc/CMV expression vector, was introduced into Jurkat cells by electroporation. Selection with G-418 led to the isolation of six neomycin-resistant clones. In parallel, the expression vector not containing the vpr insert was also introduced into Jurkat cells (mock transfection). Seven control neomycin-resistant clones were isolated. Clones obtained after transfection of pRc/CMV–vpr or pRc/CMV empty vector were screened for vpr mRNA expression by PCR analysis. Fig. 1 *A* shows the results of the PCR analysis of four representative vpr-transfected clones in which the expression of vpr transcripts was compared with that of mock-transfected clones and acutely HIV-infected T cells. In all vpr-transfected clones, full-length vpr mRNA transcripts of the expected size (319 bp) were detected after amplification with vpr-specific primers. The same transcripts were detected in acutely HIV-infected cells, but not in mock-transfected clones. The specificity of the vpr transcripts was further controlled by Southern blot analysis, using a vpr probe recognizing an internal sequence of the PCR product (data not shown).

The vpr mRNA–positive clones were subsequently characterized for the presence of vpr protein by immunoprecipitating extracts obtained from cells harvested during the logarithmic growth with an anti-vpr–specific antibody (Fig. 1 *B*). The mobility of the protein detected in the vpr clones was consistent with the 15 kD predicted size of vpr. The levels of vpr expressed in the stable transfectants approximate those obtained at early steps (3–5 d) of an acute HIV IIIB infection of Jurkat cells, as evaluated by both RNA-PCR and immunoprecipitation analyses (data not shown).

HIV-1 vpr Expression Does Not Result in a Cell Cycle Arrest in Jurkat Cells. The vpr transfectants did not show any delay in the rate of proliferation, as detected by trypan blue dye exclusion, in comparison to the parental or mock-transfected clones (data not shown). We then determined the cell cycle distribution of the parental Jurkat cells, mock-transfected, and vpr transfectants by measuring the DNA content by flow cytometry. As shown in Fig. 2, the vpr

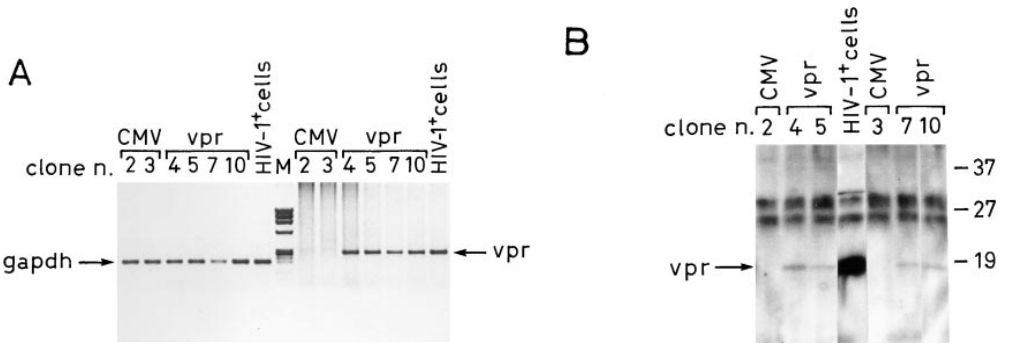

Figure 1. Detection of vpr mRNA and protein in transfected clones. (*A*) Total cellular RNA (1 μg) extracted from representative mock-transfected (*CMV2* and *CMV3*) and vpr-expressing (*vpr4, vpr5, vpr7, vpr10*) clones or from acutely HIV-infected Jurkat cells (4 d after infection) was reverse transcribed and the cDNA amplified by PCR. The PCR products were visualized by ethidium bromide staining and their size determined in comparison with a molecular weight marker (*M*). Amplification of the GAPDH mRNA was used as an internal control. (*B*) The expression of vpr protein was analyzed by immunoprecipitation with a polyclonal antibody directed against recombinant HIV-1 vpr. The immune complexes were loaded on a 18% SDS-polyacrylamide gel and transferred to a filter. The filter was then blotted using an antibody directed against an NH_2-terminal peptide of vpr, as described in Materials and Methods. Cell lysates from acutely HIV-infected Jurkat cells (7 d after infection) were used as positive controls.

transfectants exhibited a G_2/M:G1 ratio comparable to that of parental cells or mock-transfected clones. Similar results were obtained by the bromodeoxyuridine incorporation method (data not shown).

Jurkat Cell Clones Stably Expressing vpr Are Less Susceptible to Undergo Apoptosis Induced by TNF-α, Fas Triggering, and Serum Starvation. We then investigated whether the constitutive expression of vpr could modulate the capacity of Jurkat cells to undergo apoptosis. In a first set of experiments, apoptosis induced by a combined treatment with CHX and TNF-α was evaluated by fluorescence microscopy. As shown in Fig. 3, the Jurkat parental cells (*b*) as well as mock-transfected clones (*c*) displayed the typical apoptotic morphology, characterized by chromatin condensation and clumping, as early as 4 h after treatment. In contrast, a marked reduction of all apoptotic parameters and of the number of cells undergoing apoptosis was observed in vpr transfected cells (*d*). Apoptotic cell death induced by the combined treatment with CHX and TNF-α was also confirmed by flow cytometry analysis. As shown in Fig. 4, the formation of the typical broad hypodiploid DNA peak (sub G1 peak) characteristic of DNA bp fragmentation was significantly reduced in vpr transfectants as compared to parental or mock-transfected clones.

In a set of independent experiments, the number of apoptotic cells was specifically quantified by immunofluorescence after cell staining with Hoechst 33258 dye (49). As shown in Fig. 5 (*A*), only a moderate percentage of vpr-expressing cells underwent apoptosis upon addition of CHX and TNF-α (15.25% ± 1.2), whereas mock-transfected cells could be induced to undergo marked levels of apoptosis (55.62% ± 2.05).

To gain some insight into the possible cell target controlled by the vpr, we investigated the effect of the constitutive expression of this protein on the apoptosis induced by stimuli acting through different signalling pathways (i.e., Fas triggering and 24 h of serum starvation). As shown in Fig. 5 (*B* and *C*), a marked reduction in the percentage of cells undergoing apoptosis was detected in all the vpr transfectants after Fas stimulation (12.0% ± 1.13 versus 55.2% ± 2.4) or serum starvation (7.75% ± 1.12 versus 23.0 ± 1.10), thus suggesting that putative mediator(s) common to all these patterns are probably affected as result of the vpr expression.

Vpr Antisense Phosphorothioate Oligodeoxynucleotide Renders vpr-expressing Cells Highly Susceptible to Apoptosis Induced by CHX and TNF-α. To assess whether the decreased susceptibility of vpr-expressing cells to undergo apoptosis was due to a direct effect of the endogenously expressed vpr, we investigated the effect of antisense phosphorothioate oli-

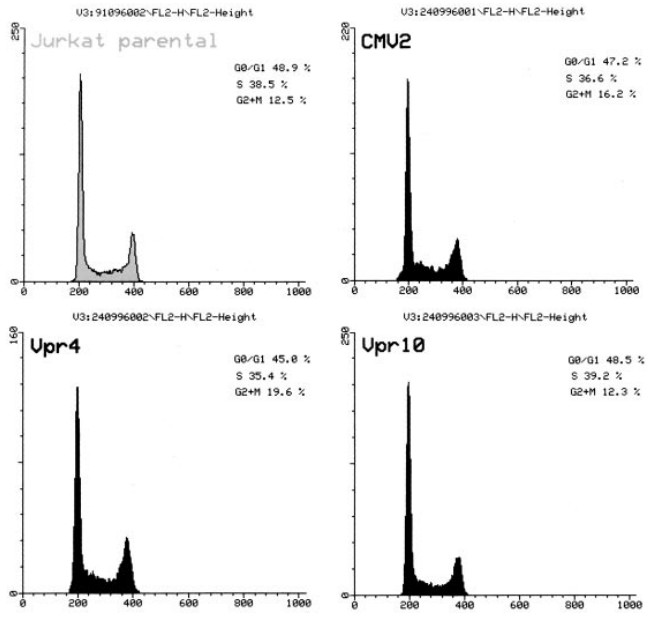

Figure 2. Cell cycle analysis of vpr-transfected cells. DNA analysis was performed by PI staining. Histograms of flow cytometry analysis of DNA content in Jurkat parental cells, a mock-transfected clone (*CMV2*), and two vpr-expressing clones (*vpr4* and *vpr10*) are shown. The abscissa indicates red fluorescence (linear scale). In the ordinate, the relative cell number is indicated. The percentage of cells in the different phases of the cell cycle was obtained as described in Materials and Methods. One representative experiment out of four is shown. Similar results were obtained with all the other vpr-expressing and control clones (data not shown).

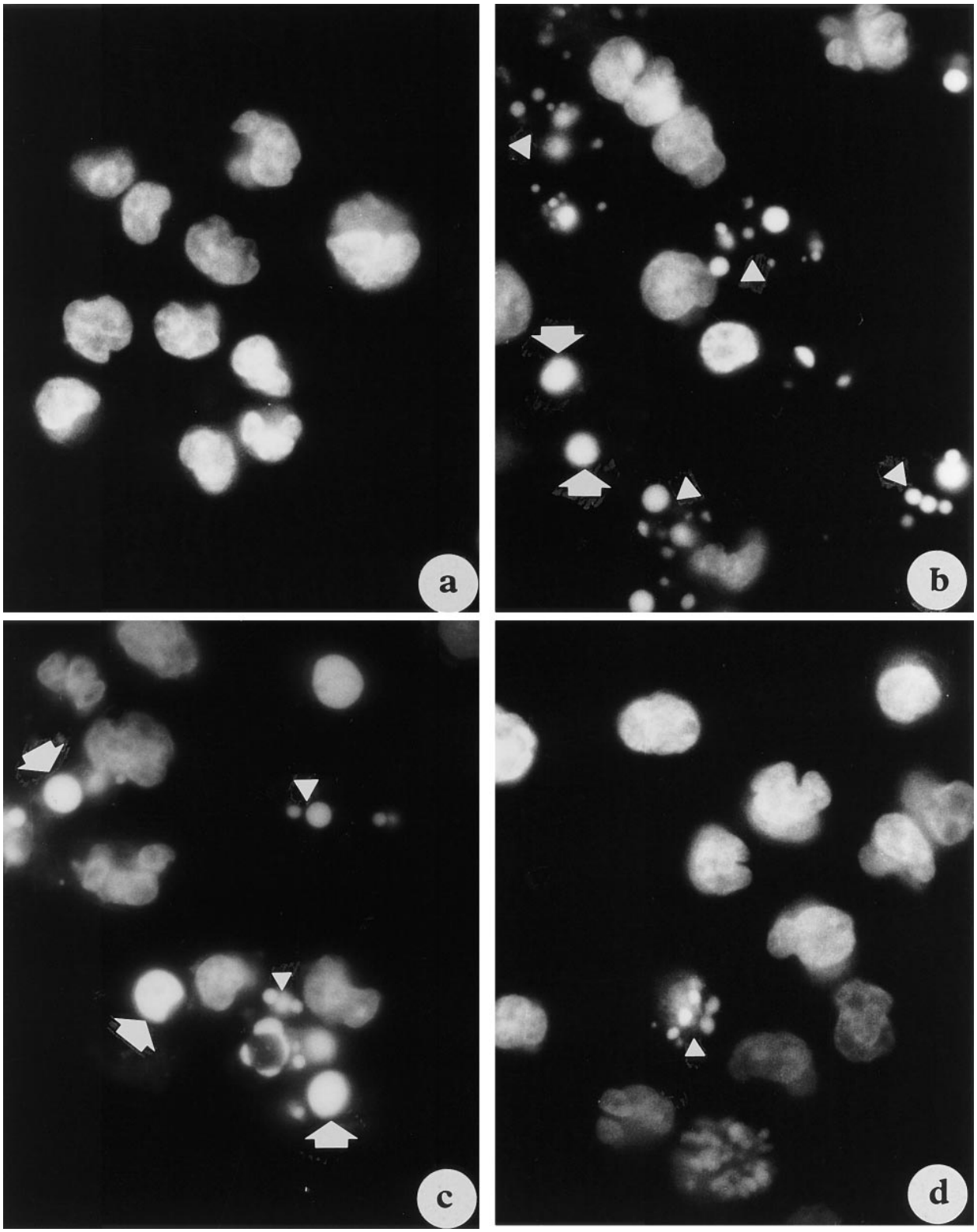

Figure 3. Morphological analysis of apoptosis induced by CHX and TNF-α. Jurkat parental cells (*b*), a mock-transfected (clone CMV2; *c*) and vpr-expressing (clone vpr4; *d*) cells were stimulated with CHX (25 μg/ml) for 2 h and then treated with TNF-α (12.5 ng/ml) for an additional 2 h. Apoptosis was evaluated by fluorescence microscopy using the chromatin dye Hoechst 33258. The apoptotic morphology is clearly visible in *b* and *c*, in which a high number of cells show the typical condensation (*arrows*), fragmentation, and clumping (*arrowheads*) of chromatin, as compared to unstimulated parental cells (*a*). In contrast, the vpr-expressing cells (*d*) are protected from apoptosis. All vpr transfectants and mock-transfected clones displayed a similar behavior (data not shown).

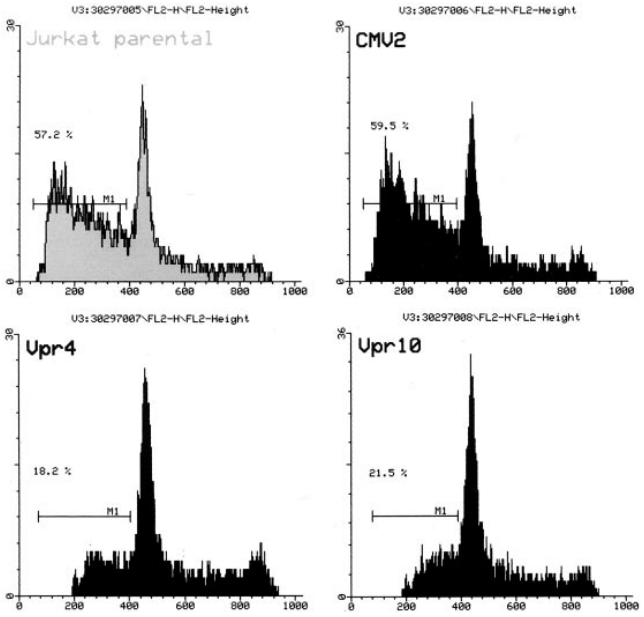

Figure 4. Flow cytometry analysis of cells stimulated with CHX and TNF-α. Cells were stimulated as described in the legend to Fig. 3, stained with PI, and then analyzed by a FACScan® flow cytometer. Bars (*M1*) indicate cells in the hypodiploid compartment. A representative experiment out of four is shown. The percentage of spontaneous apoptosis was <1%.

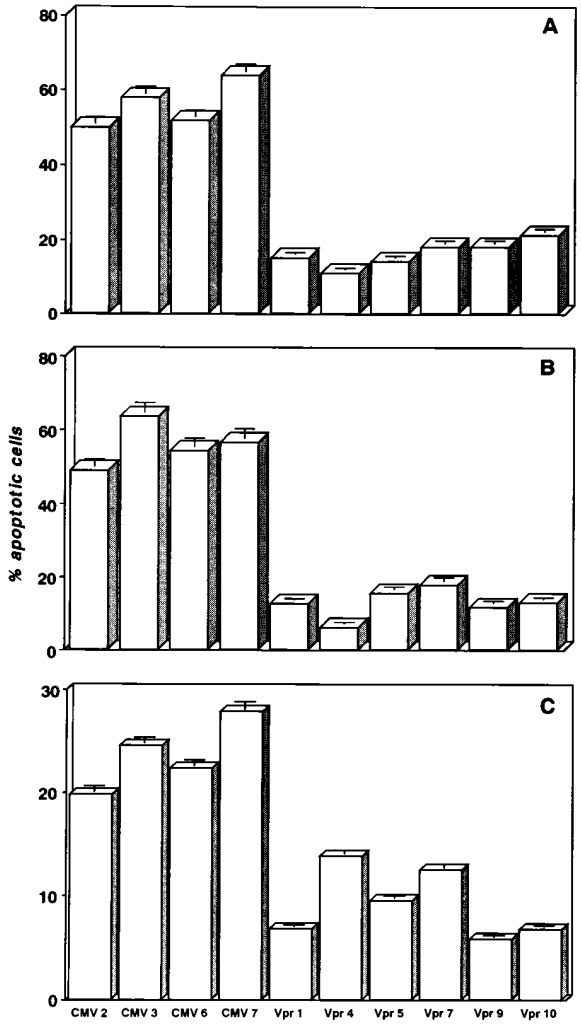

Figure 5. Quantitation of the extent of apoptosis induced by CHX and TNF-α, Fas triggering, and serum starvation. Vpr-expressing (*Vpr*) and mock-transfected (*CMV*) clones were stimulated to undergo apoptosis with a combination of CHX and TNF-α as described in the legend to Fig. 3 (*A*), antibody to Fas (50 ng/ml for 4 h; *B*) and serum starvation (24 h; *C*). Quantitative evaluation of apoptosis was performed by fluorescence microscopy. The values of spontaneous apoptosis were in the range <1% up to a maximum of 2% for all clones. Results, expressed as percentage of apoptotic cells, were obtained by counting at least 400 cells at a 500 magnification in a blind code. Values represent the mean ± standard errors of the results obtained in six independent experiments. The values of the vpr clones were statistically significant (i.e., $P < 0.01$ versus the values of the control clones).

godeoxynucleotide targeted to vpr, which had been tested for their efficacy in blocking the expression of the vpr protein (data not shown). As shown in Fig. 6, only a moderate percentage of vpr-expressing cells underwent apoptosis upon addition of CHX and TNF-α (16.01%) as compared to mock-transfected cells (49.20%). When antisense oligonucleotide was added to the cell culture before the apoptotic stimulus, the susceptibility of vpr-expressing cells to undergo apoptosis was completely restored (45.97%). The increased percentage of apoptotic cells in the presence of antisense oligonucleotide was not due to oligonucleotide-induced cellular toxicity, as assessed by trypan blue dye exclusion (data not shown). Moreover, the addition of the control sense oligonucleotide did not exert any effect. Likewise, the addition of either type of oligonucleotides to mock-transfected clones did not exert any effect on the percentage of cells undergoing apoptosis. Similar results were obtained with two other vpr-expressing clones (clone 9 and 10) and mock-transfected clones (clone 2 and 5) after induction of apoptosis by CHX and TNF-α or Fas triggering (data not shown). These results strongly indicate that the resistance to the induction of apoptosis of vpr-expressing cells is directly linked to vpr expression.

Analysis of bcl-2 and bax Proteins in vpr-expressing Cells. Lastly, we investigated the possibility that vpr expression was associated with any modulation of genes known to be involved in the regulation of apoptosis. The intracellular levels of bcl-2 and bax proteins were investigated by using a specific flow cytometry analysis. As shown in Fig. 7 (*top*), a 30% increase in the expression of bcl-2 protein was observed in vpr-expressing clones as compared to the mock-transfected or parental counterparts. We also investigated the expression of bax, a cellular protein that binds to bcl-2 and suppresses its ability to block apoptosis. The intracellular levels of bax were significantly lower in vpr transfectants (a 20% decrease) as compared to mock-transfected clones (Fig. 7, *top*).

The expression of bcl-2 and bax proteins in vpr-expressing clones as well as in mock-transfected and parental cell clones was further analyzed by immunoblotting with spe-

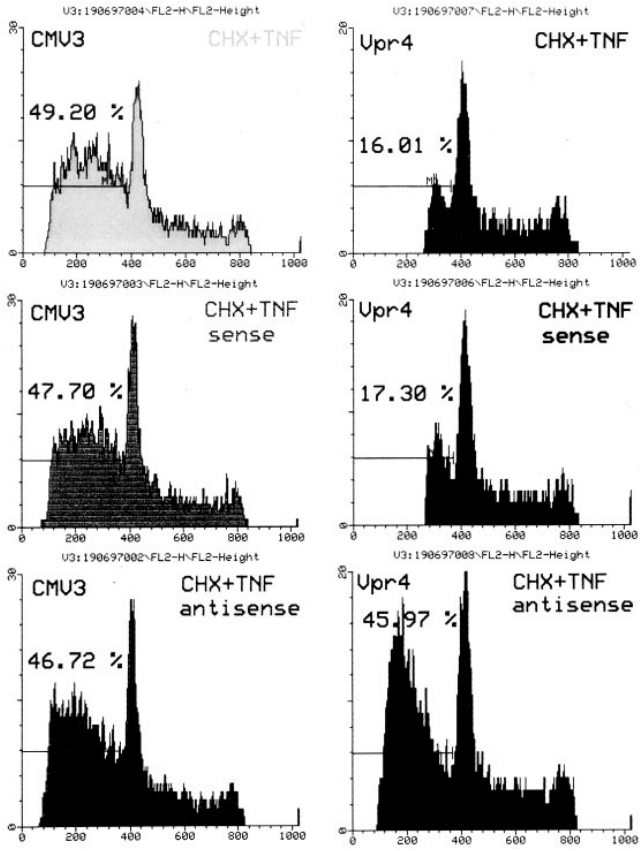

Figure 6. Flow cytometry analysis of mock-transfected and vpr-expressing cells treated with phosphorothioate oligodeoxynucleotides targeted to vpr. vpr-expressing cells (clone 4) and mock-transfected cells (clone 3) were treated with phosphorothioate sense or antisense oligonucleotides as described in Materials and Methods and then stimulated with CHX and TNF-α. Apoptosis was evaluated by flow cytometry as described in the legend to Fig. 4. Similar results were obtained in two additional experiments.

cific antibodies. The results of a representative experiment shown in Fig. 7 (*bottom*) indicates that vpr expression results in an upregulation of bcl-2 and in a parallel decrease in the levels of bax.

Discussion

The immunopathogenesis associated with HIV-1 infection is characterized by several functional abnormalities and by a progressive depletion of $CD4^+$ T lymphocytes. However, the mechanisms by which HIV kills $CD4^+$ T cells have not yet been elucidated (50). Several mechanisms have been proposed to explain the decline of $CD4^+$ T cells. Some of them may be related to direct HIV-mediated cytopathic effects such as syncytia formation and single-cell killing (51). Other mechanisms may involve a series of immunological phenomena, such as elimination of HIV-infected CD4-positive cells by HIV-specific CTLs or by nonspecific cytotoxic mechanisms (52), autoimmunity (53), or by anergy induced by inappropriate signaling (54). Some studies have shown that the cytopathic effect of HIV in mononuclear cell populations is associated with apoptosis and that this phenomenon occurs in both $CD4^+$ and $CD8^+$ T lymphocytes of HIV-infected individuals (55–57). Abnormally high levels of apoptotic cells are also detected in lymph nodes of HIV-infected individuals (58). In general, intensity of apoptosis correlates with the state of activation of the lymphoid tissue and not with stage of disease or viral burden (58). Recent findings have indicated that apoptosis is rarely observed in productively infected cells in the lymph nodes, whereas it occurs predominantly in bystander uninfected cells (59). This finding argues in favor of indirect mechanisms for $CD4^+$ T cell depletion, rather than direct killing of these cells by the virus. Consistent with this hypothesis, it has been recently reported that coculture of HIV-infected with uninfected cells results in cell death by apoptosis of uninfected cells (60).

We have reported herein that the constitutive expression of the HIV-1 vpr protein in Jurkat cells results in an impaired capacity of undergoing apoptosis induced by different stimuli. The specific role of vpr in the cell protection from apoptosis is demonstrated by the finding that antisense vpr oligonucleotides render vpr-expressing cells as susceptible to apoptosis induced by CHX and TNF-α as the control clones. Our data may provide an explanation for the apparent lack of apoptosis in HIV-infected cells. Moreover, the antiapoptotic activity of vpr may have additional implications in the pathogenesis of HIV infection. Activation of an endogenous cell suicide program represents a host strategy to limit viral spread (25). In regards to HIV, it is of interest to mention that Chinnaiyan et al. (61) very recently suggested that apoptosis may serve as a beneficial host mechanism to limit viral spread. In particular, these authors demonstrated that inhibition of proapoptotic ICE-like proteases results in enhanced viral production in T lymphocytes exposed to HIV-1. Many viruses encode genes inhibiting the host cell death apparatus (25–35). Although there is evidence that the HIV-1 tat protein is capable of influencing apoptosis (36–42), the vpr protein exhibits the unique property of being carried into the virions. This suggests that vpr plays an important role in the first steps of infection, when the regulatory proteins (tat and rev) are not yet present. It should be noted that the vpr levels expressed in our transfectants closely resemble those detectable at early stages (3–5 d) of acute infection of Jurkat cells. At later stages of infection, however, higher levels of vpr expression are observed, in association with the peak of HIV replication (a few days before cell death).

In this study, we also reported that the constitutive expression of vpr in Jurkat cells did not interfere with the progression in the cell cycle. In fact, the vpr transfectants exhibited a G2/M:G1 ratio comparable to that of parental or mock-transfected cells. Several reports have demonstrated that the expression of vpr at high levels, by either transient or proviral DNA transfection, resulted in the inhibition of the cell cycle (18–22). The apparent discrepancy between our results and those from others could be explained by either the differences in the cell types and in the

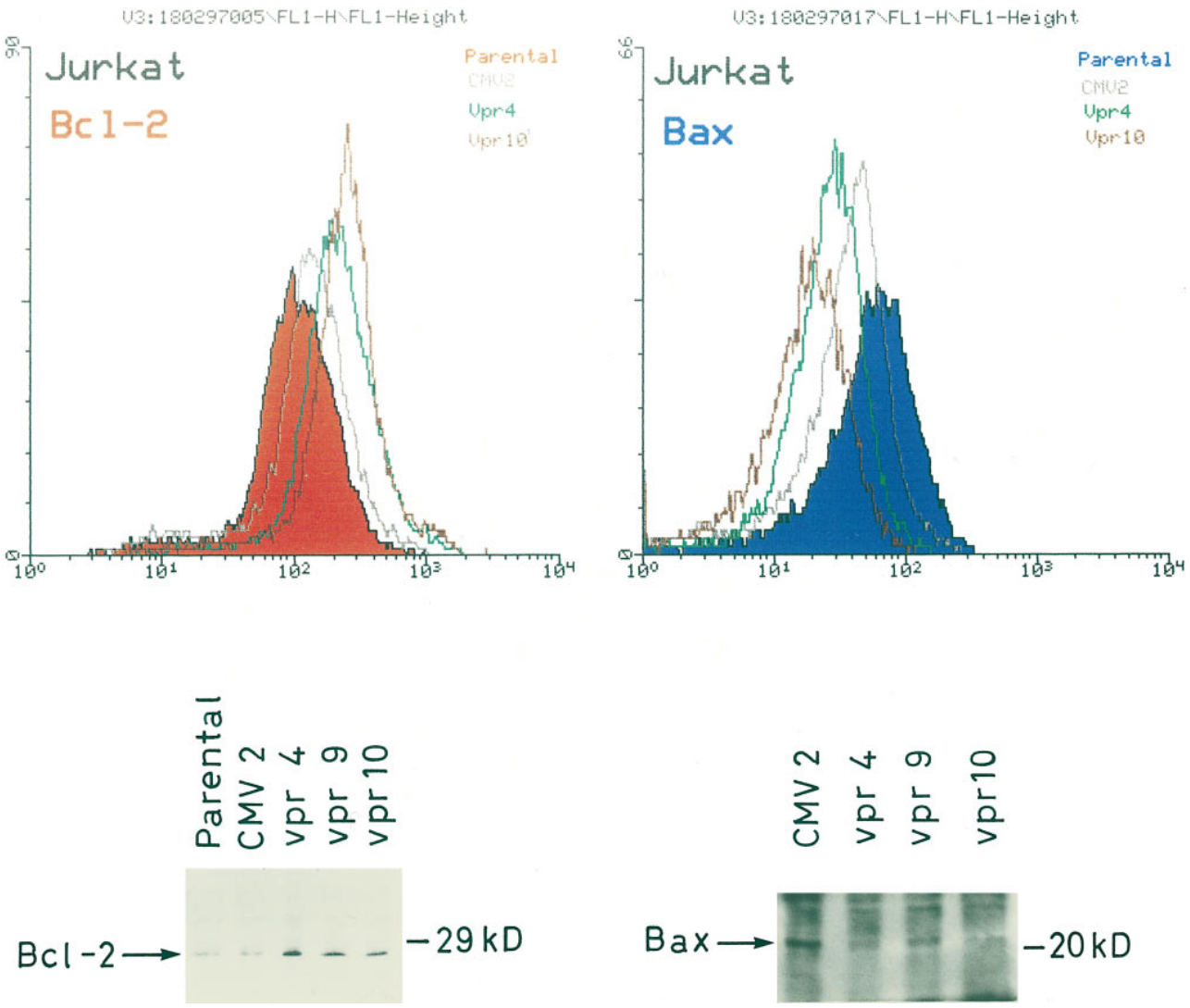

Figure 7. Effect of vpr on the expression of bcl-2 and bax proteins. The intracellular expression of bcl-2 and bax proteins was measured in vpr-expressing and mock-transfected clones by flow cytometry analysis (*top*) and by immunoblotting (*bottom*), as described in Materials and Methods. Quantitative evaluation of bcl-2 and bax proteins was performed by converting the logarithmically amplified signals obtained by flow cytometry into values on a linear scale. In vpr-expressing cells, the extent of bcl-2 increase and bax decrease with respect to mock-transfected control clones was of 30 and 20%, respectively. These changes are the mean of three different experiments performed with three representative vpr-expressing clones, Jurkat parental cells, and two mock-transfected clones. The increase of bcl-2 and the decrease of bax were highly reproducible in all the experiments. The statistical significance of the values obtained was calculated by using the Kolmogorov-Smirnov test. A $P < 0.001$ was considered significant. The levels of bax expression in the Jurkat parental cells was proved indistinguishable from those detected in mock-transfected clones (data not shown).

experimental approaches used for achieving vpr expression or by a dual effect of vpr. Notably, none of these papers demonstrated a direct correlation between the concentration of vpr achieved into the cells and the extent of cell cycle inhibition. Furthermore, the direct effect of vpr as inhibitor of cell cycle progression is supported only by experiments in which proviruses, either wild type or mutated, have been used. For instance, in the experiments published by Bartz et al. (22) with the Jurkat cells, the G_2 arrest was observed in 59% of cells infected with vpr wild-type provirus (multiplicity of infection 10) under experimental conditions in which 100% of the cells were infected. Furthermore, treatment of PBMCs with crude baculovirus supernatants containing vpr resulted in inhibition of cell proliferation in 50–60% of the cells, depending on the stimulus used for triggering T cell proliferation (44). In light of all these data, it cannot be ruled out that vpr exerts a dual role depending on its concentration. Interestingly, it has been reported by Rogel et al. (18) that vpr expression in Jurkat cells, obtained by infection at high multiplicity of infection with vpr^+ and vpr^- pseudotypes, resulted in cell cycle arrest 12 d after infection, when the vpr expression achieved was probably very high. A recent study by Stewart et al. (43) showed that the ability of vpr to arrest cells in the G_2 phase finally resulted in the induction of apoptosis without the addition of exogenous stimuli. Notably, a vpr-mutated provirus

could also induce apoptosis, albeit at lower levels than the wild-type provirus, indicating that other viral factors are involved in the induction of apoptosis (43). More recently, it has been reported that A1.1 cells treated with crude baculovirus supernatants containing vpr induced apoptosis in 50% of the cells. In contrast, the same vpr treatment resulted in the inhibition of apoptosis induced by anti-CD3 antibody (44). This regulation of apoptosis was linked to vpr suppression of NF-κB activity, via the induction of IκB. Nevertheless, it should be noted that although the antiapoptotic activity of exogenous vpr was studied in A1.1 cells, the NF-κB–binding activity and IkB mRNA expression was investigated in vpr-transfected rhabdomyosarcoma cells and PBMCs, respectively (44). All this, together with the fact that the crude baculovirus supernatants contained unknown amounts of vpr, did not allow any definitive conclusions. Our study is the first report on the inhibitory effect of vpr, constitutively expressed at low levels in T cells, on apoptosis. Although further studies are needed to clarify the role of vpr in the pathogenesis of HIV infection, we envisage the following scenario, taking into account all our results and data from the literature: (*a*) the vpr protein imported into the target cell could exert an antiapoptotic function in the very early steps, thus allowing the establishment of the infection; (*b*) the low levels of vpr expressed in the first days after infection could continue to exert their antiapoptotic activity; and (*c*) the high expression of vpr at late steps of infection could result in cell growth arrest, subsequently leading to cell death. This scenario would take into account different aspects of the HIV life cycle. In fact, depending on the physiological status of the target cell and probably on many other factors (such as multiplicity of infection, virus strain, cellular cofactors), a different expression of the vpr protein could be obtained in different cells, thus allowing two possible outcomes of the infection: (*a*) virus-induced cytophatic effect at high vpr concentration; and (*b*) survival of some infected cells at low vpr concentration. Notably, a dual effect depending on the concentration of a viral protein has been previously described for the HIV Tat protein (42). The fact that the same protein can determine different effects in the host cell depending on its concentration would provide the virus with a versatile and advantageous tool for driving the cell fate from survival (persistence) to cell death (cytophatic effect).

The nature of the vpr-induced signals involved in the vpr action remains to be elucidated. The fact that vpr protects Jurkat cells from different apoptotic stimuli, either protein synthesis dependent (Fas) or independent (TNF-α and serum starvation), suggests that this protein acts on an upstream step common to all these inducers. Our data indicate that vpr may act, at least in part, by regulating the balance of cellular genes (bcl-2 and bax) involved in the regulation of apoptosis.

Altogether, our results suggest that low levels of the endogenous vpr protein can interfere with the physiological turnover of T lymphocytes at early stages of virus infection, thus facilitating HIV persistence and, subsequently, viral spread. We suggest that the vpr protein could exert its action upstream of the cell survival machinery, thus functioning as an endogenous regulator of the cell fate.

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