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Mitochondrial Membrane Hyperpolarization Hijacks Activated T Lymphocytes Toward the Apoptotic-Prone Phenotype: Homeostatic Mechanisms of HIV Protease Inhibitors

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A decrease of mitochondrial membrane potential has been hypothesized to be a marker of apoptotic cells, including activated T lymphocytes. It was recently demonstrated that HIV protease inhibitors, independently from any viral infection, can hinder lymphocyte apoptosis by influencing mitochondrial homeostasis. To analyze the mechanisms underlying these effects, a specific study was undertaken in both resting and activated human PBL exposed to either receptor (e.g., anti-Fas)- or nonreceptor (e.g., radiation)-mediated apoptotic stimuli. T cell activation was found to be accompanied by a significant increase in mitochondrial membrane potential, or hyperpolarization, which was undetectable in resting cells. We also detected apoptotic hindering by HIV protease inhibitors only in activated T lymphocytes. This was apparently due to the ability of these drugs to block activation-associated mitochondria hyperpolarization, which, in turn, was paralleled by an impairment of cell cycle progression. Remarkably, protease inhibitors also prevented zidovudine-mediated mitochondrial toxicity. Finally, HIV-infected cells from naive patients behaved identically to activated T cells, displaying hyperpolarized mitochondria, while lymphocytes from patients under highly active antiretroviral therapy (which included HIV protease inhibitors) seemed to react as resting cells. Altogether these results clearly indicate that the hyperpolarization state of mitochondria may represent a prerequisite for the sensitization of lymphocytes to the so-called activation-induced cell death. They also suggest that HIV protease inhibitors, by interfering with induction of the mitochondrial hyperpolarization state, can result in cell survival even independent of any viral infection. ***The Journal of Immunology,*** **2003, 170: 6006–6015.**

The complex cascade of events involving proapoptotic signals, including activation of several apoptosis-specific proteases, i.e., caspases, depends on the type of the trigger that activates the process (1). A hypothesis regarding two different apoptotic pathways leading to activation of cell-specific programs has recently been proposed. These two pathways reflect different initiation patterns: receptor dependent or independent (2, 3). Both pathways, however, converge toward specific mitochondrion activity (3). In particular, it has been suggested that changes of mitochondrial membrane potential $(\Delta\Psi)$[2] play a key role in apoptotic cascade. Given the important role of apoptosis in the pathogenesis and progression of HIV infection, several studies have specifically focused on apoptosis mechanisms in both HIV-infected and uninfected $CD4^+$ cells (4, 5). In particular, a major role of the mitochondria in the process of CD4 T cell death has been suggested (6, 7). In fact, the reduction of $CD4^+$ cell loss by apoptosis has been considered to be an important aspect of immune reconstitution under highly active antiretroviral therapy (HAART) (8, 9). This is a clinical approach that involves, among others, drugs of different nature, such as HIV reverse-transcriptase inhibitors, e.g., zidovudine (AZT), and HIV protease inhibitors (PIs). Those most commonly used are nelfinavir, indinavir (ind), saquinavir (saq), and, very recently, lopinavir (lop). Their combined activity leads to viral load lowering as well as to reduced cell loss. Several lines of evidence have schematically indicated that AZT may be an apoptotic inducer (10), while PIs can be considered as apoptosis-hindering drugs (9, 11, 12). More specifically, the use of AZT, although of relevance in the control of the progression of the disease through direct activity on viral replication, was demonstrated to be per se cytotoxic to the immune system cells (13). In particular, some studies clearly indicated that AZT was irreversibly incorporated into mitochondrial DNA inducing mitochondrial dysfunction by acting on polymerase γ and inhibiting oxidative phosphorylation (14, 15). This resulted in apoptotic cell death process (10, 15). In contrast, some in vitro and ex vivo studies suggested that PIs, independent from any viral infection, were able to inhibit PBMC loss and to restore impaired T cell proliferative response (16). Accordingly, apoptotic cell death of both HIV-infected and uninfected cells was apparently inhibited. The mechanisms underlying this activity are, however, still controversial (11, 17). One of these was hypothesized to involve a target activity of PI drugs toward T cell mitochondria (18, 19). In the present work, based on data from the above literature, we analyzed the mechanisms involved in the subcellular effects of PIs and AZT, both considered as mitochondriotropic drugs. We found that: 1)

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[2] Abbreviations used in this paper: $\Delta\Psi$, mitochondrial membrane potential; AZT, zidovudine; DHR 123, dihydrorhodamine 123; HAART, highly active antiretroviral therapy; HD, healthy donor; hsp, heat shock protein; ind, indinavir; JC-1, 5–5′,6–6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; lop, lopinavir; PARP, poly(ADP-ribose) polymerase; PI, protease inhibitor; *p*NA, *p*-nitroanilide; ROI, reactive oxygen intermediate; saq, saquinavir; TRAIL, TNF-related apoptosis-inducing ligand.

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unlike resting cells, mitochondrial membrane was hyperpolarized once lymphocytes were activated and that this was a prerequisite for apoptotic cell death susceptibility; 2) PIs significantly inhibited both cell cycle progression and apoptosis in activated lymphocytes, exerting a target effect on mitochondria, i.e., by stabilizing activated T cell mitochondrial $\Delta\Psi$ (which, in the presence of PIs, remained similar to that detectable in resting cells). Finally, and according to the above results, 3) these drugs also prevented AZT-mediated mitochondrial toxicity.

Materials and Methods

Isolation and activation of PBL

Human PBL from healthy donors (HD) and HIV patients were isolated from freshly heparinized blood through a Ficoll-Hypaque density-gradient centrifugation and washed three times in PBS, pH 7.4 (Lympholyte-H; Cedarlane Laboratories, Hornby, Ontario, Canada). PBL were subcultured in 25- or 75-cm^2 Falcon plastic flasks at a density of $\sim 1 \times 10^6$ cells/ml in RPMI 1640 (Life Technologies, Milan, Italy) containing 15% FCS (Flow Laboratories, Irvine, Scotland), 1% nonessential amino acids, 5 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 mg/ml) at 37°C in a humidified 5% CO_2 atmosphere. For PBL activation, purified T cells were activated for 72 h with PHA (2 μg/ml; Boehringer Mannheim, Milan, Italy) and IL-2 (60 IU/ml; Life Technologies).

Patients

Twelve HIV-positive patients (6 males and 6 females with a mean age of 37 $\pm$ 7 years) provided informed consent and were included in this study. Patients were excluded if they were receiving corticosteroids or antineoplastic chemotherapy or if they were suffering from opportunistic infections at the time of evaluation. Six patients were under HAART for at least 1 year and had an HIV viral load below the limit of quantitative evaluation ($<$50 copies/ml) and a CD4$^+$ cell count of 430 $\pm$ 28/mm^3. The HAART regimen included AZT and lop boostered by a baby dose of ritonavir (20). Six patients naive for antiretroviral therapy with high values of HIV viral load (171,350 $\pm$ 95,320 copies/ml) and low CD4 counts (120 $\pm$ 89 mm^3 were also considered in this study. HIV viral load was estimated by branched-chain DNA assay (Chiron, Emeryville, CA) with a limit of detection below 50 copies/ml. Six healthy volunteers were used as controls.

Drugs and chemicals

For in vitro treatments (see below), the following PIs, kindly provided as pure lyophilized powders by the manufacturers, were used: saq (by Roche Registration, Welwyn, Garden City, Hertfordshire, U.K.), ind (by Merk Sharp & Dome, Hotteson, Hertfordshire, U.K.), and lop (by Abbott Laboratories, Queenborough, U.K.). For apoptosis studies, the following chemicals were used: 1) TNF-α (Sigma-Aldrich, St. Louis, MO); 2) anti-CD95/Fas-triggering mAb (clone CH11; Upstate Biotechnology, Lake Placid, NY); 3) anti-CD95/Fas-neutralizing mAb (clone ZB4; Upstate Biotechnology); 4) TNF-related apoptosis-inducing ligand (TRAIL; Alexis, San Diego, CA); 5) AZT (by Sigma-Aldrich). Moreover, 6) UV radiation B (UVB, 1200 J/m^2) as nonreceptor-mediated physical apoptotic inducer was also considered.

In vitro treatments

PI treatments. Lyophilized ind, saq, and lop were dissolved in sterile DMSO. Isolated human lymphocytes (resting or activated) were treated with different concentrations of various PIs (0.1, 1, 10, 100 nM and 1, 10, 100 μM) added daily for 72 h. As a control, an equal volume of DMSO was added to lymphocytes. Subsequently, lymphocytes were treated with different apoptotic stimuli (see below).

Apoptosis induction. The following proapoptotic treatments were considered: 1) CD95 triggering: 500 ng/ml of an anti-human Fas IgM mAb (clone CH11; Upstate Biotechnology) was added to lymphocytes and cultured cells (6×10^5 cells/ml) for 48 h; 2) TNF-α treatment: 6×10^5 cells/ml were treated with cycloheximide (Sigma-Aldrich) and 50 IU/ml of TNF-α (Sigma-Aldrich), as stated elsewhere (20); 3) TRAIL triggering: human lymphocytes were incubated with soluble TRAIL (50 ng/ml) in the presence of 2 μg/ml of enhancer Ab (TRAIL soluble human Kit; Alexis) for 48 h; 4) UVB radiation: 6×10^5 cells/ml were exposed to UVB irradiation in PBS using Philips TL 20 W/12 lamp. To eliminate UVC radiation, a Kodak filter (Kodacell TL 401) with an OD of less than 0.4 for wavelengths below 285 nm was used and was placed on the petri dishes during exposure. In these conditions, the UVB radiant flux density to the cells was 1200 J/m^2, as verified by an Osram centra UV meter. UVB radiation was also performed in the presence of neutralizing anti-human Fas IgG1 mAb (clone ZB4; 250 ng/ml; Upstate Biotechnology). Apoptosis was evaluated up to 72 h after radiation.

Analytical cytology

Cell cycle analysis. Cell cycle progression analysis of resting and IL-2/PHA-activated T cells, in the presence or absence of various PIs, was performed by flow cytometry, as previously described (21). Cells were fixed and permeabilized with ice-cold ethanol for 30 min and, after this time, washed twice with PBS. DNA staining was performed by incubating cells at 37°C in PBS containing 40 μg/ml propidium iodide and 0.4 mg/ml DNase-free RNase (type 1-A). Samples were analyzed collecting FL2 red fluorescence in a linear scale at above 620 nm. The percentage of cells in the different phases of the cell cycle was determined by ModFIT software analysis. Apoptotic cells and debris were excluded by these analyses.

Evaluation of cell surface receptors. The surface expression of molecules associated with T cell phenotype (CD4), T cell activation (CD69, CD38, and HLA-DR), and cell death (CD95/Fas, 55-kDa TNFR1a, and 75-kDa TNFR1b) was verified by flow cytometry on resting and activated human lymphocytes. To this purpose, mAbs directly conjugated to fluorochromes PE, FITC, or PerCP to human CD4, CD95, CD38, HLA-DR (BD Biosciences, Mountain View, CA), and TNFR1a (55 kDa) and TNFR1b (75 kDa) (Caltag Laboratories, Burlingame, CA) were used. Appropriate fluorochrome-conjugated Igs were used as negative controls. For cytometric analyses of data regarding surface molecule expression, only electronically gated CD4$^+$ lymphocytes were considered. No significant variation in the expression of these receptors was induced by PI treatments.

Apoptosis evaluation. Quantitative evaluation of apoptosis was performed by using the following flow and static cytometry methods: 1) TdT incorporation of labeled nucleotides into DNA strand breaks (TUNEL-FITC; Boehringer Mannheim). Cells fixed with 4% formaldehyde in PBS for 15 min were prepared according to manufacturer instructions; 2) double staining using annexin V-FITC apoptosis detection kit (Eppendorf, Milan, Italy). This technique allows cells that have lost membrane integrity (and therefore considered necrotic) to show red staining with propidium iodide (40 μg/ml) throughout the nucleus and to be easily distinguished from the living cells; 3) staining with chromatin dye Hoechst (Molecular Probes, Eugene, OR), as previously described (22). For cytometric analyses of data regarding apoptosis, only electronically gated CD4$^+$ lymphocytes were considered.

$\Delta\Psi$ and mass. The $\Delta\Psi$ of control and treated cells was studied by using the JC-1 probe. Following this method, cells were stained with 10 μM of 5–5′,6–6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Molecular Probes), as previously described (23). Iodide probe 3,3′-dihexyloxacarbocyanine (Molecular Probes) and tetramethylrhodamine ester (Molecular Probes) were also used to confirm data obtained by JC-1. Because the probes yielded overlapping results, only data obtained with JC-1 are reported. As a methodological control, cells were treated with increasing concentrations (from 0.1 to 10 μg/ml) of K$^+$ ionophore valinomycin (Sigma-Aldrich) and then stained with JC-1 (data not shown). For the analysis of mitochondrial mass, cells were incubated at 37°C for 30 min with 5 μM nonylacridine orange (Molecular Probes). After washing, samples were immediately analyzed by flow cytometry. To verify cell viability, parallel tubes were incubated with PI (40 μg/ml) for 15 min at 37°C (23).

Reactive oxygen intermediate (ROI) production. Cells (5×10^5) were incubated in 495 μl of HBSS, pH 7.4, with 5 μl of dihydrorhodamine 123 (DHR 123; Molecular Probes) in polypropylene test tubes for 15 min at 37°C (final concentration: 10 μM). DHR 123 is a dye freely diffusing into cells, oxidized primarily by H_2O_2 in a myeloperoxidase-dependent reaction to green fluorescence. As DHR 123 is accumulated by mitochondria, the production of ROI at the mitochondrial level can be detected, as stated elsewhere (24).

Quantitative analysis of Bcl-2 family proteins and heat shock protein (hsp) 70

Flow cytometry. Cells were first pelleted and fixed in 70% ice-cold methanol. After washings with cold PBS, samples were stained with mAb to Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or to hsp70 (Transduction Laboratories, Lexington, KY) or with rabbit polyclonal Abs to Bax,

Bcl-x_L (Santa Cruz Biotechnology). Negative controls were incubated with mouse IgG1 or total rabbit serum. After 1 h at 4°C, samples and isotypic controls were incubated for 30 min at 37°C with FITC-labeled anti-mouse or FITC-labeled anti-rabbit (Sigma-Aldrich). After washings, cells were analyzed on a flow cytometer.

Western blot. Aliquots of total protein extracts (30 μg) from cells after different treatments were size fractioned by 10–12% SDS-PAGE and electroblotted overnight at 4°C onto nitrocellulose membranes. Filters were incubated with primary Abs against Bcl-2, Bax, Bcl-x_L (Santa Cruz Biotechnology), or hsp70 (Transduction Laboratories). Detection was achieved using HRP-conjugated secondary Ab (anti-mouse, anti-rabbit) and by ECL detection system (Amersham-Pharmacia, Arlington Heights, IL).

Activation of caspases and poly(ADP-ribose) polymerase (PARP) cleavage. For detection of the active form of caspases 8, 9, and 3, colorimetric protease assay kits (Chemicon International, Temecula, CA) were used. Proteins obtained from cytosolic extracts (50/200 μg) were incubated with 200 μM of IETD-*p*-nitroanilide (*p*NA) (for caspase 8), LEHD-*p*NA (for caspase 9), or DEVD-*p*NA (for caspase 3). The assay was based on spectrophotometric detection of the chromophore *p*NA after cleavage from the labeled substrates. *p*NA light emission was quantified using a microtiter plate reader at 405 nm. Comparison of the absorbance of *p*NA from apoptotic samples with an uninduced control allows determination of the fold increase in caspase activity (25). To reveal p58 PARP fragment, control and apoptosis-triggered human activated lymphocytes were pelleted and fixed by using fix and perm reagent (Caltag Laboratories). After washings, cells were stained with specific rabbit polyclonal Ab to p85 PARP fragment (Promega, Milan, Italy). After 1 h at 4°C, samples and isotypic control were incubated for 30 min at 37°C with FITC-labeled anti-mouse (Sigma-Aldrich). Cells were then analyzed on a flow cytometer.

Data analysis and statistics

All samples were analyzed with a FACScan cytometer (BD Biosciences) equipped with a 488 argon laser. At least 20,000 events were acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software. Calculation of fluorescence (expressed as median value of fluorescence emission curve) was conducted after conversion of logarithmically amplified signals into values on a linear scale, and the statistical significance was calculated by using the parametric Kolmogorov-Smirnov (K/S) test. Statistical analysis of apoptosis data was performed by using Student's *t* test or one-way ANOVA by using Statview program for Macintosh. All data reported in this study are the mean of at least four separate experiments ± SD. Only *p* values of <0.01 were considered as significant.

Results

Evaluation of cytotoxicity and cell cycle effects of PIs

To assess the effects of ind, saq, and lop on cell viability, we first determined the toxicity of the drugs in freshly isolated human peripheral T lymphocytes. No cytotoxic effects were revealed up to 1 μM of PI concentration (annexin V/propidium iodide double negative). By contrast, higher PI concentrations (from 10 to 100 μM) led to a dose-dependent increase in necrotic cell death (annexin V negative/propidium iodide positive) in human peripheral lymphocytes.

Experiments were also conducted to evaluate cell cycle features of T cells during activation in the presence of various PIs. As expected, while no S phase cells were detectable in resting T lymphocytes either in the presence or absence of PIs, after 72-h activation with IL-2/PHA, a percentage of cells in S phase (11.0% ± 3.1) or in G_2 M phase (12.9 ± 3.3) was observed, according to mitotic stimulation exerted by PHA. Notably, in the presence of PIs, these percentages dropped to values comparable to those found in resting cells (below 1%). These cytostatic effects were accompanied by a decreased

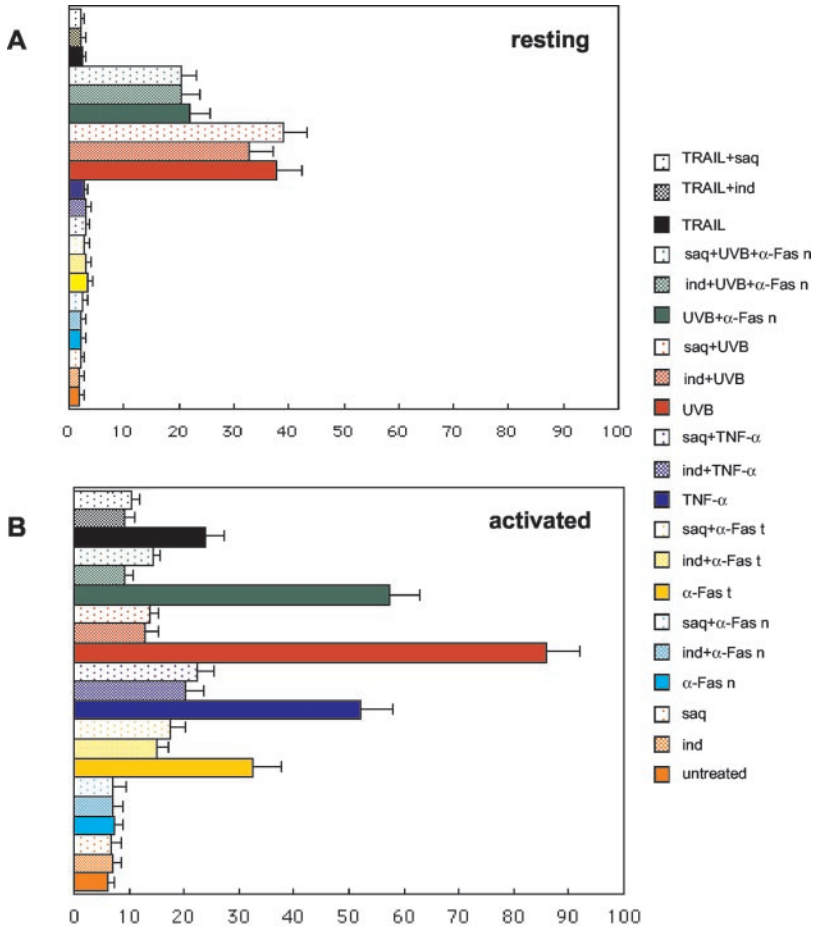

FIGURE 1. Effects of PIs on human resting and activated lymphocyte apoptosis. *A*, PIs are ineffective on apoptosis of resting lymphocytes. Percentage of apoptotic cells (as shown by annexin V/propidium iodide double staining) in resting human PBL from HD. Note that: 1) no significant receptor-mediated apoptosis was detectable; 2) resting cells were susceptible to UV radiation (red bars), but no protective activity was exerted by PIs (red/dotted bars); and 3) anti-Fas-neutralizing mAb (α-Fas n, green bars) significantly protected resting T cells from UV-induced apoptosis (red bars). *B*, PIs hinder apoptosis of activated lymphocytes. Percentage of apoptotic cells (as revealed by annexin V/propidium iodide double staining) in IL-2/PHA-activated PBL from HD. Note: 1) the different susceptibility to various pro-apoptotic stimuli, and 2) that PIs were capable of significantly reducing apoptosis ($p < 0.01$ for each apoptotic stimulus vs the same treatment performed in the presence of PIs) independently from the stimulus considered.

spontaneous apoptotic rate that dropped significantly from 12 ± 1.5% during IL-2/PHA activation to 2.3 ± 1.2% in the presence of PIs (mean value of the results obtained with various PIs). Finally, as expected, cell surface activation molecules tested, e.g., CD95 and CD69, were typically increased in PHA/IL-2-treated cells. Strikingly, the same molecules were not affected by the exposure to various PIs: median values, in terms of fluorescence intensity detected, remained substantially the same as those found in control activated cells (e.g., median values for CD95 were 41.4 ± 4.3 in activated cells and 42.1 ± 5.9 in ind-treated activated cells; for CD69, the values found were 12.3 ± 2.6 and 12.7 ± 4.3, respectively).

Antiapoptotic effects of PIs in resting and activated human lymphocytes

We investigated whether various PIs were able to prevent apoptosis in both resting and activated human lymphocytes. Healthy donor-derived PBL were incubated with ind, saq, or lop (all used at the same concentration, 100 nM) in the presence (activated lymphocytes) or absence (resting lymphocytes) of IL-2 and PHA. After 72 h, cells were exposed to various apoptotic stimuli, as stated above, i.e., Fas-triggering, TNF-α, TRAIL, and UVB radiation.

Resting cells

As expected, resting lymphocytes were generally resistant to induction of apoptosis (Fig. 1*A*). In particular, they were completely refractory to receptor-mediated triggering, with apoptosis values similar to those of spontaneous apoptosis found in control samples (below 10%). In contrast, they were partially susceptible to UVB-induced apoptosis (Fig. 1*A*). However, PI treatment did not induce any protective effect, and the apoptotic values remained unchanged compared with control samples (only representative results obtained with ind and saq have been included in Fig. 1). Furthermore, because UV radiation was hypothesized to activate the Fas pathway via receptor aggregation and subsequent recruitment of the death adaptor molecule Fas-associated death domain protein/MORT1 (26), we also irradiated cells in the presence of neutralizing anti-Fas Ab. In fact, we found that Fas-neutralizing Ab was capable of significantly inhibiting radiation-induced apoptosis, but also that PIs were ineffective and apoptosis occurred normally in resting lymphocytes.

Activated cells

In contrast, results obtained in activated human lymphocytes (Fig. 1*B*) were the following: 1) apoptotic proneness of activated lymphocytes was significantly higher compared with

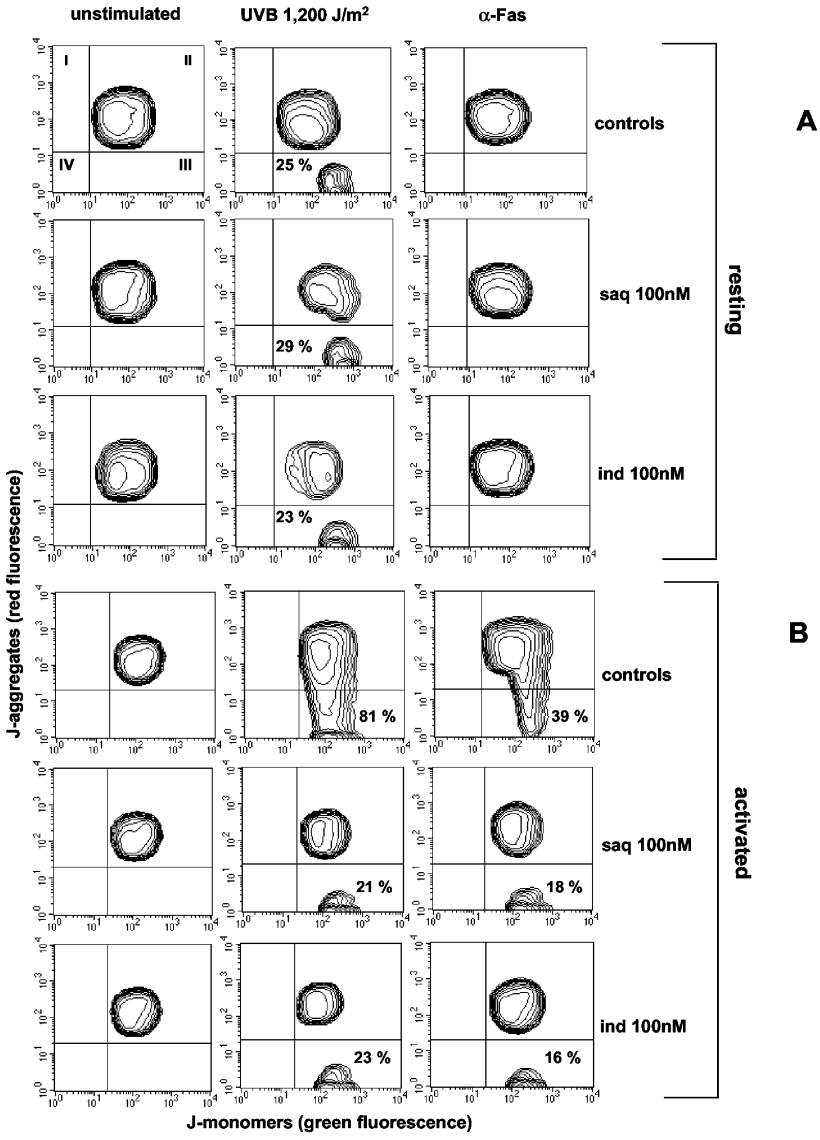

FIGURE 2. PIs and $\Delta\Psi$. *A*, Effects of PIs on mitochondrial integrity of resting lymphocytes exposed to apoptotic stimuli. Cytofluorometric analysis of $\Delta\Psi$ (performed by using JC-1) in resting PBL from HD. Control (*left panels*), UV-treated (*middle panels*), and anti-Fas-triggered (*right panels*) cells are shown. Contour plots clearly show that cells with depolarized mitochondria can be detected in UV-treated cells only (III quadrant), while anti-Fas treatment was ineffective (*right panels*). Notably, saq and ind had no effects. *B*, Effects of PIs on mitochondrial integrity of activated lymphocytes exposed to apoptotic stimuli. Cytofluorometric analysis of $\Delta\Psi$ in activated PBL from HD. Analyses of control (*left panels*), UV-treated (*middle panels*), and anti-Fas-triggered (*right panels*) cells show that UV- and Fas-mediated mitochondrial membrane depolarization (*upper row*) was powerfully counteracted by PIs (*middle* and *bottom rows*). Note the decreased percentage of cells with depolarized mitochondria shown in the III quadrant. One experiment representative of four is shown.

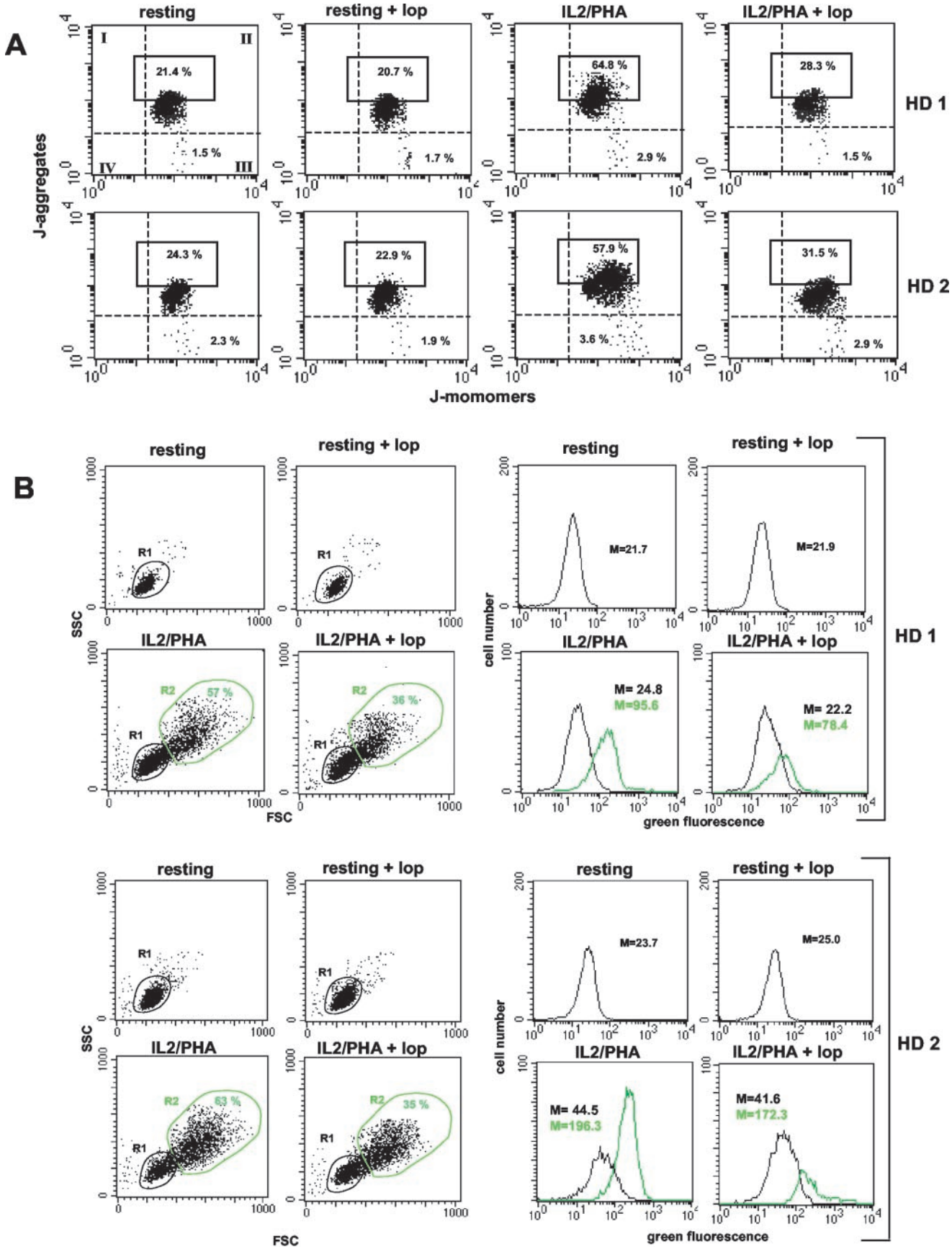

FIGURE 3. $\Delta\Psi$ (*A*) and ROI production (*B*) during T cell activation. *A*, Qualitative and quantitative cytofluorometric analyses of the $\Delta\Psi$ in both resting and activated (IL-2/PHA for 72 h) lymphocytes with or without lop, as detected by using the JC-1 probe. J-aggregates (red fluorescence) typically increased when mitochondrial membrane becomes hyperpolarized (numbers in the boxed areas represent the percentage of cells with hyperpolarized mitochondria). *B*, Cytofluorometric analysis of ROI production performed by using DHR 123 in resting and IL-2/PHA-activated lymphocytes in the presence (or absence) of lop. Values reported represent the median values of the fluorescence intensity histograms. Note that after activation: 1) a modification of physical

resting cells, and 2) receptor-mediated stimuli (TNF-α, TRAIL, anti-Fas) were characterized by lower values of apoptosis (54, 22, and 34%, respectively) compared with radiation (>80%); more importantly, 3) a significant decrease in cell loss by apoptosis was found in activated human T lymphocytes pre-exposed to PIs before exposure to various apoptotic inducers (Fig. 1*B*). In particular: 1) the protection conferred by PIs (ind, saq, and lop, 100 nM) was, regardless of the stimulus used, significant in receptor-mediated (anti-Fas, TNF-α, TRAIL) as well as in receptor-independent (UVB and UVB + anti-CD95/Fas-neutralizing Ab) apoptosis, and 2) no significant difference was found between various PIs in terms of antiapoptotic activity. Specifically, the mean values obtained from quantitative cytofluorometric analysis (conducted by pooling together the results obtained with ind, saq, and lop) indicated that PIs were capable of significantly reducing TNF-α-mediated apoptosis ($-70.1 \pm 7\%$), Fas-mediated apoptosis ($-55.4 \pm 8\%$), TRAIL-induced apoptosis ($-49.0 \pm 6\%$), and UVB-mediated apoptosis ($-79.6 \pm 8\%$) in activated T cells. In Fig. 1, only the results obtained with ind and saq have been reported.

PI and $\Delta\Psi$

The literature suggests that one of the main markers of apoptosis-associated mitochondrial modification leading to cell death may be a significant decrease occurring in $\Delta\Psi$, i.e., the loss of $\Delta\Psi$ (27). Specific flow cytometry experiments were therefore conducted on PI-pretreated lymphocytes (both resting and activated) to evaluate the possible activity of various PIs on mitochondrial integrity and function. Representative results obtained after Fas triggering or UV radiation are reported in Fig. 2, *A* and *B*, referring to resting and activated lymphocytes, respectively. Cells with depolarized mitochondria are those included in the III quadrant of the contour plots. According to apoptosis data (see Fig. 1, *A* and *B*), by evaluating the percentages of cells with depolarized mitochondria, we found that pretreatment for 72 h with 100 nM of each PI in this study considered was capable of significantly decreasing the percentage of cells showing loss of $\Delta\Psi$. In particular, receptor-mediated apoptosis (by anti-Fas, TNF-α, TRAIL) induced a significant mitochondrial membrane depolarization in activated lymphocytes only (39%), while nonreceptor-mediated stimuli (radiation) induced the loss of $\Delta\Psi$ in both resting (25%) and activated (81%) T cells. Interestingly, PI pre-exposure exerted a significant protection from $\Delta\Psi$ decrease in activated cells only, while radiation-induced mitochondrial depolarization in resting cells was not prevented. Summarizing, the analysis of the percentages of cells with depolarized mitochondria, with or without PI pretreatment (performed pooling together the results obtained with ind, saq, and lop), clearly indicated a significant ($p < 0.01$) decrease in the percentage of T cells showing $\Delta\Psi$ loss ($-72 \pm 5\%$ in UV radiation-treated cells and $-51 \pm 4\%$ in receptor-mediated apoptotic cells). In Fig. 2, *A* and *B*, only results obtained with ind and saq are shown.

To investigate the mechanisms underlying the different susceptibility to the antiapoptotic activity exerted by PIs in activated rather than resting lymphocytes, an analysis of the $\Delta\Psi$ state before and after activation in six different healthy donors was conducted. Strikingly, as shown in Fig. 3*A* (in which only two representative healthy donors of six analyzed are shown), an increased mitochondrial transmembrane potential, namely a hyperpolarization of the inner mitochondrial membrane, was detected after activation in a significant percentage of lymphocytes. In fact, after 72-h exposure to PHA and IL-2, an increase of fluorescence emission in FL2 channel was observed (corresponding to J-aggregates, which typically increase when mitochondrial membrane becomes more polarized). A specific quantitative analysis indicated that a high percentage of T cells with hyperpolarized mitochondria (mean value obtained pooling together the results achieved from T cells of six different HD = $66.8 \pm 7.7\%$) was detectable after activation (see the boxed area of the plotted graph, high red fluorescence). This phenomenon was detected in lymphocytes from all HD considered in this study. Most importantly, the activation of lymphocytes in the presence of various PIs did not determine any increase in $\Delta\Psi$, which remained very similar to that observed in resting cells (Fig. 3*A*). This stabilizing activity of PIs resulted in fact in a low percentage of activated T cells with hyperpolarized mitochondria ($29.3 \pm 3\%$, mean value obtained by pooling together data concerning saq, ind, and lop, compared with $26.1 \pm 1.8\%$ found in resting control T cells). According to literature suggesting that T cells from HIV^+ patients are constitutively activated and apoptosis prone (28), we found a significantly higher percentage of cells with hyperpolarized mitochondria in freshly isolated T cells from naive HIV patients with respect to T cells from healthy donors ($\Delta = +27 \pm 3\%$, mean value from six patients compared with six healthy donors). Conversely, lymphocytes from patients under HAART (six patients selected, as stated in *Materials and Methods*) displayed percentages of cells with hyperpolarized mitochondria not significantly different to those found in HD.

Because mitochondria hyperpolarization has been related to ROI hyperproduction (29), quantitative analysis of ROI generated during lymphocyte activation in the presence or absence of various PIs (saq, ind, and lop) was also performed by flow cytometry. In Fig. 3*B*, the increased ROI production detected using DHR 123 in activated T cells compared with resting cells can be observed (compare resting and IL-2/PHA dot plots and histograms). In particular, this increased ROI production was overall detected in a subset of lymphocytes that increased their physical parameters, i.e., their volume (dot plots, R2 region), as detected by fluorescence intensity histograms (green lines). Importantly, PI drugs, e.g., lop, were capable of significantly hindering both ROI production (green lines, see median values in the histograms) and changes in physical parameters described above and detectable in activated T lymphocytes (dot plots, R2 region). Hence, PIs (saq, ind, and lop) were able to significantly prevent either mitochondria hyperpolarization (Fig. 3*A*) or oxidative imbalance (Fig. 3*B*) associated with IL-2/PHA activation. Only data obtained with lop, considered as representative results, are shown in Fig. 3.

Bcl-2 family proteins and hsp70

Given the hypothesized role played by Bcl-2 family proteins in the modulation of apoptosis in HIV-infected cells (30) as well as in

parameters of T cells was detected (dot plots, R2 region); 2) an increased ROI production was found (green lines in the histograms); and 3) PI exposure, e.g., lop, was capable of partially preventing changes in physical parameters (dot plots; see percentages shown in R2) and ROI production (see median values and green lines in the histograms). Significant changes ($p < 0.001$) were found in: 1) resting vs activated T lymphocytes and 2) IL-2/PHA-activated vs activated lymphocytes in the presence of lop. Only the results from two representative healthy donors of four are shown. Similar data were obtained by using saq and ind. Note that the percentage of cells with spontaneously depolarized mitochondria is very low in both resting and activated T cells (numbers in the III quadrant).

maintaining mitochondrial homeostasis (31), the expression of Bcl-2, Bax, and Bcl-x_L in activated lymphocytes in the presence of various PIs was investigated. However, our results clearly demonstrated no significant variation in the expression of these proteins. In particular, PI administration did not induce significant variations in median values as detected by flow cytometry analyses. For instance, basal values (expressed as median value of the fluorescence intensity histogram) of Bcl-2 (15.63 ± 0.98), Bax (8.66 ± 1.1), and Bcl-x_L (12.6 ± 1.3) detectable in activated T cells remained substantially unchanged after PI administration (e.g., with ind: Bcl-2 = 15.29 ± 1.8; Bax = 8.54 ± 1.4; and Bcl-x_L = 12.3 ± 2.0). Similarly, hsp70, hypothesized to have a role in the modulation of mitochondrial-mediated apoptosis (32, 33), did not undergo significant changes in the presence of PIs, as detected by both flow cytometry and Western blot analyses (not shown). For instance, basal values (expressed as median value of the fluorescence intensity histogram) of hsp70 detectable in activated T cells (9.39 ± 1.9) remained substantially unchanged after ind administration (9.23 ± 2.4). Similar results were obtained with saq and lop (data not shown).

Effects of PIs on AZT subcellular activity

Previous reports suggested that one of the most important drugs in the management of AIDS, i.e., AZT, can be considered an apoptotic inducer in activated lymphocytes (10). More interestingly, it was also suggested that mitochondria could represent an important intracellular target of this drug (34–37). We thus decided to evaluate whether mitochondrial toxicity and subsequent apoptosis induced by AZT could be counteracted by PI subcellular activity. We used AZT as mitochondriotropic drug and apoptotic inducer in untreated or PI-treated IL-2/PHA-activated lymphocytes. Results reported in Fig. 4, *A* and *B* (because no significant differences were detected among various PIs, only representative results obtained with ind are shown), clearly showed that: 1) PIs prevented AZT-induced apoptosis (Fig. 4*A*) and 2) this protection was mediated by a protective effect exerted on mitochondrial homeostasis (Fig. 4*B*, *left panels*, III quadrant). In addition, our results also show that: 3) AZT sensitized lymphocytes to receptor-mediated apoptotic triggering, e.g., by anti-Fas (Fig. 4*A*) and that 4) this sensitization was paralleled by an increase in the percentage of cells with depolarized mitochondria (Fig. 4*B*, *right panels*). Overall, these findings suggest that: 1) AZT-induced mitochondrial-mediated cell death of activated lymphocytes can be significantly prevented by PIs (the mean decrease of apoptosis exerted by PIs was 74 ± 12%, pooling results from saq, ind, and lop) (Fig. 4*A*); 2) the percentage of cells with depolarized mitochondria can be significantly lowered by pretreatment with PIs (data obtained by using saq, ind, and lop pooled together showed a mean decrease of 68 ± 8%) (Fig. 4*B*); 3) a significant correlation exists between the two events ($r = 0.978$, $p < 0.001$); and, finally, 4) cumulative synergic proapoptotic effects of AZT and various proapoptotic stimuli, e.g., by anti-Fas (Fig. 4*A*), can be counteracted by different PIs. In Fig. 4, *A* and *B*, considering the similar behavior exerted by PIs toward various apoptotic stimuli, only the more extensively studied physiologic stimulus, i.e., anti-Fas, is shown.

Mitochondria downstream events: caspase activation and PARP cleavage

We then investigated the involvement of caspase cascade in the protective effects exerted by PIs on apoptosis. A series of experiments aimed at the detection of caspase 8, 9, and 3 activity were conducted. Fig. 5*A* clearly shows that the inhibition of Fas-induced apoptosis by PIs is associated with a significant ($p < 0.01$) decrease in caspase 9 and caspase 3 activity. By contrast, importantly, no significant change was observed in the mitochondria upstream caspase, i.e., caspase 8, activity. In Fig. 5*A*, because results overlapped, only the effects exerted by ind and saq are shown. Finally, results obtained by analyzing PARP (Fig. 5*B*) clearly indicated that Fas-induced PARP cleavage was significantly impaired by various PIs (Fig. 5*B*, *right panel*; only results obtained with ind and saq are shown). For example, Fas-induced PARP cleavage in activated T cells was significantly ($p < 0.01$) reduced by PI pretreatment (data obtained by using saq, ind, and lop pooled together

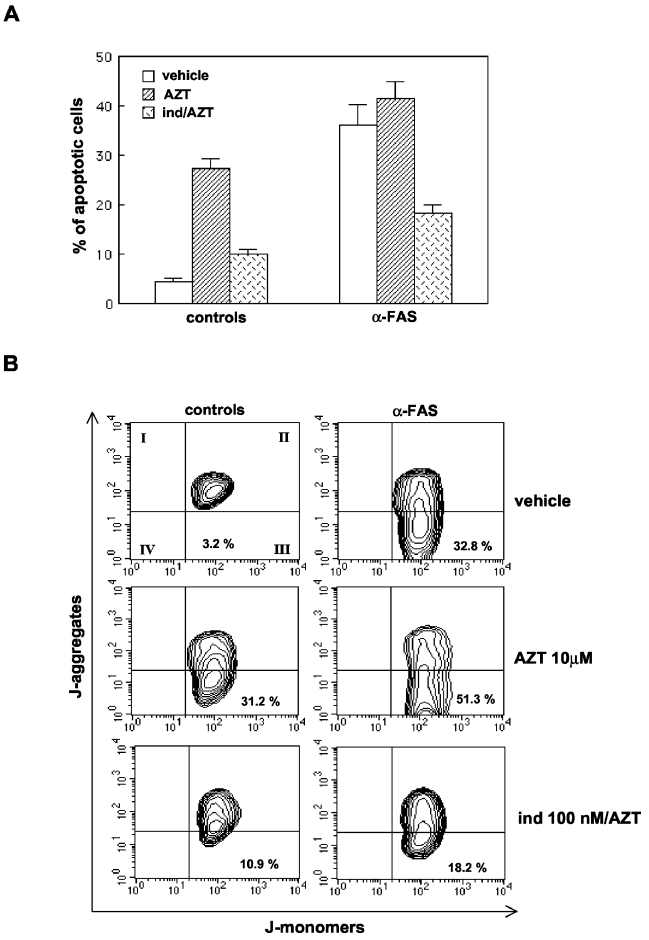

FIGURE 4. PIs protect from subcellular effects of AZT. *A*, Apoptosis. Quantitative evaluation of proapoptotic effects of AZT in untreated and anti-Fas-treated activated PBL from HD, as revealed by double staining with annexin V/propidium iodide. Note that: 1) AZT given alone was capable of inducing apoptosis (hatched column on the *left*); 2) combined treatment of AZT with another proapoptotic stimulus, i.e., anti-Fas, led to a significantly increased percentage of apoptotic cells (hatched column on the *right*); 3) the PI ind was able to significantly ($p < 0.01$) decrease the percentage of apoptotic cells in both AZT- and anti-Fas/AZT-treated activated T cells (dotted columns). Value of $p < 0.01$, AZT vs ind + AZT. *B*, Mitochondria. Cytofluorometric analysis of $\Delta\Psi$ in activated PBL from HD. Control (*left panels*) and anti-Fas-treated cells (*right panels*) are shown. PIs, e.g., ind, were able to counteract AZT-induced mitochondrial alterations, i.e., depolarization. Note that cells with depolarized mitochondria (percentages in the III quadrant, *middle row*) were significantly ($p < 0.01$) reduced by ind pre-exposure (percentages in the III quadrant, *bottom row*). Similar results were obtained by using saq and lop. Data from one HD representative of four are shown.

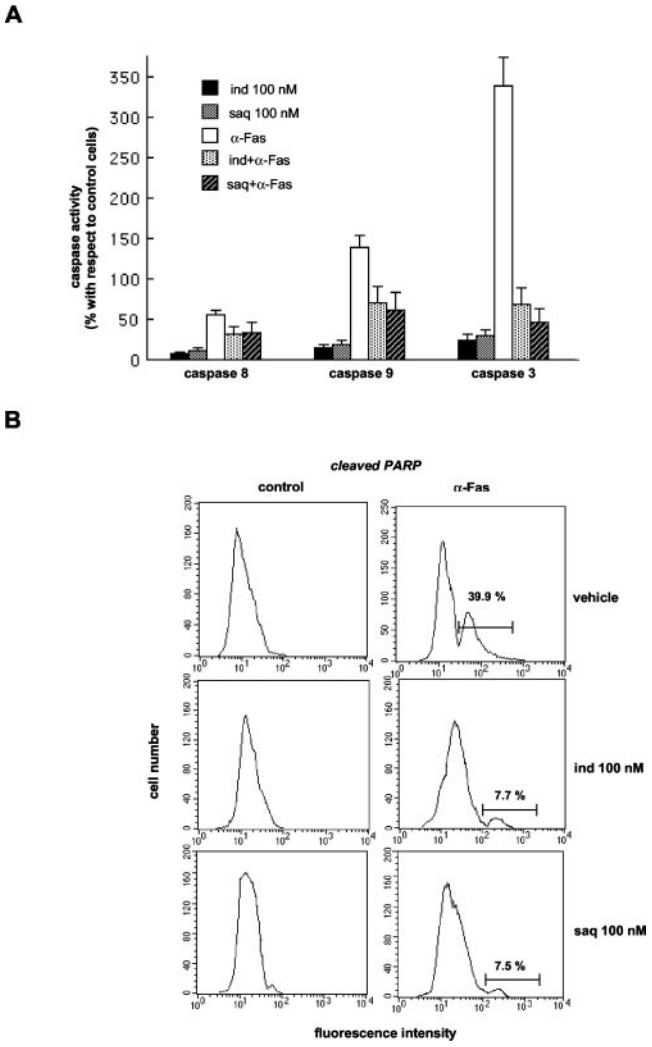

FIGURE 5. Analyses of apoptotic cascade. *A*, Caspase activity. The activity of caspases 8, 9, and 3 in anti-Fas-treated activated T cells in the presence or absence of PIs saq and ind obtained by a colorimetric assay. Reported values were obtained by considering the difference between caspase activity found in treated cells with respect to untreated control cells. Note that the activity of caspase 8 (upstream to mitochondria) remained unaffected by the presence of PIs, while caspases 9 and 3 appeared significantly inhibited. Value of $p < 0.01$, anti-Fas vs ind + anti-Fas and anti-Fas vs saq + anti-Fas, for both caspases 9 and 3. *B*, PARP cleavage. Fas-induced PARP cleavage (*right panels, upper row*) was significantly ($p < 0.01$) impaired by the presence of both ind (*right panels, middle row*) and saq (*right panels, bottom row*). The numbers in the pictures indicate percentages of cells with cleaved PARP, as revealed by the cytofluorometric analysis. Because of similar results obtained by using lop, only results obtained by ind and saq are shown. One representative experiment of four is shown.

showed a decrease of $-80.8 \pm 4.7\%$), according to data obtained on caspase 3 activity (in Fig. 5*A*, only the results obtained with ind and saq are shown).

Discussion

Cell loss in the immune system of HIV-infected patients has been described as a sort of apoptotic proneness leading to the depletion of $CD4^+$ cells (5). More recently, the widespread use of PIs in these patients has led to new findings in this field. In fact, antiviral activity was accompanied by immune reconstitution. This attracted the attention of physicians examining the immune pharmacological activity of these drugs (9, 38, 39). Conflicting results have, however, been reported to date regarding the role of PIs in determining lymphocyte fate in patients under HAART (11, 16, 40, 41).It was, for example, suggested that HAART and specifically PIs were capable of reducing apoptosis of $CD4^+$ cells independently from plasma viremia (9, 34, 40, 42, 43). In contrast, it was also hypothesized that some PI, e.g., ind, can inhibit ex vivo cell cycle progression of PBMC from HIV-infected and uninfected individuals, but without affecting apoptosis (44). In our conditions, together with inhibition of cell cycle progression, a clear antiapoptotic activity was found with three different PIs, i.e., ind, saq, and lop (the last very recently introduced in clinical practice and, to the best of our knowledge, used for the first time in experimental analyses on apoptosis), but in IL-2/PHA-activated human T cells only. This inhibition was significantly exerted (50% or more) toward those apoptotic stimuli that involve cell surface receptors such as TNF family receptors, as well as toward a physical agent such as UV radiation (>75%). By contrast, resting T cells remained unaffected. Because of the importance of mitochondria, in particular of $\Delta\Psi$, in apoptotic cascade (27), we analyzed in detail the changes occurring in mitochondria of T cells during activation.

It is well known that both receptor-dependent and independent apoptosis generally converge toward mitochondrially driven cascade (2, 3). This is characterized, as a late event, by the loss of $\Delta\Psi$ with release of apoptogenic factors, i.e., cytochrome *c* and apoptosis-inducing factor, and involves a plethora of downstream events such as apoptosome formation, caspase 9 and caspase 3 activation, and PARP cleavage (3). This cascade can be regulated by Bcl-2 family proteins that can impair, e.g., by Bcl-2 or Bcl-x_L, or favor, e.g., by Bax, apoptotic process. In the present study, we found a selective intracellular influence of PIs on earlier events occurring in the main supervisors of this cascade, i.e., the mitochondria (45). In particular, a characteristic increase of $\Delta\Psi$ was observed in $CD4^+$ lymphocytes during activation compared with resting conditions. This hyperpolarization phenomenon, previously described by other authors, has been hypothesized to represent a very early change occurring in mitochondria during apoptosis (29, 46). Furthermore, recent results also seem to suggest that apoptosis proneness can be exogenously modulated, e.g., by cytokines, and that this increased susceptibility to apoptosis can be associated with an increased $\Delta\Psi$ (47, 48). Accordingly, in our context, hyperpolarization state appeared to be a prerequisite for susceptibility to apoptosis of IL-2/PHA-activated T cells. In fact, higher levels of intracellular ROI in activated T cells paralleled the hyperpolarization state of mitochondrial membrane. In other words, T cell activation, mitochondria hyperpolarization, and increased ROI production seem to be related events sensitizing IL-2/PHA-activated lymphocytes to apoptotic cell death. The $\Delta\Psi$ loss, previously described as the typical marker of the execution phase of apoptosis (27), can thus be considered as a later event. It is also possible to hypothesize that the antiapoptotic activity exerted by PIs might be due to a target-stabilizing effect of these drugs on $\Delta\Psi$. In fact, the antiapoptotic effect exerted by PIs was higher in the case of those stimuli, which mainly act via oxidative stress (e.g., TNF-α and UVB radiation). Furthermore, after PI exposure, no signs of increased $\Delta\Psi$ were found in activated T cells. This might be suggestive of a specific antiapoptotic effect of PI drugs on those cells that increase their $\Delta\Psi$ as an earliest event, while they are ineffective toward those cell types that do not display this characteristic mitochondrial state. In fact, in the case of resting lymphocytes, PI antiapoptotic activity was undetectable in radiation-induced apoptosis. This

is the unique stimulus able to induce apoptosis in resting cells, i.e., in which no mitochondrial hyperpolarization was detectable.

Other results reported in the present work and regarding the apoptotic cascade and its features are consistent with this hypothesis. In fact, we have observed that mitochondria downstream events are also counteracted by PIs, i.e., $\Delta\Psi$ loss, caspase 9 function (the mitochondrial associated caspase), and caspase 3 activity as well as PARP cleavage. By contrast, caspase 8, an upstream caspase typically associated with CD95/Fas-mediated signals, remained unaffected. Interestingly, in the presence of PIs, no change was found in the expression of those regulatory molecules able to positively or negatively influence mitochondrial proapoptotic activity (Bcl-2, Bcl-x_L, Bax, and hsp70). This could suggest that, unlike other drugs that infer mitochondrial proapoptotic modifications via up (or down)-regulation of Bcl-2 family proteins (49), PIs might exert a direct target activity on mitochondria. Finally, the cytotoxic activity of the drug AZT, a known apoptotic inducer, was also fully counteracted by PIs. Because AZT is a mitochondria-targeting drug (14, 15, 34, 36), this may support the above hypothesis that modulation of PBL apoptosis by PIs might be exerted by a specific target effect on mitochondria.

Taken together, these findings, along with the literature (16, 18, 19), suggest that the effects of PIs on lymphocyte survival may be due to an HIV-independent activity on the main subcellular supervisor of suiciding cells, i.e., the mitochondrion. In contrast, because HIV-induced apoptosis of lymphocytes has been shown to be accompanied by increased ROI production (6), results reported in this work might also indicate that PIs can exert an important ROI scavenging activity in HIV-infected cells. Finally, results on the subcellular mechanisms of PIs in T cells might provide additional information of relevance for the use of these drugs in both infectious and noninfectious immunological diseases.

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