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Azidothymidine and Interferon-α Induce Apoptosis in Herpesvirus-associated Lymphomas[1]

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ABSTRACT

Lymphoproliferative diseases that occur in immunocompromised patients are frequently associated with herpesviruses. These patients often fare poorly after treatment with conventional chemotherapy. We reported previously that patients with AIDS-related Burkitt's lymphoma (BL) responded to parenteral azidothymidine (AZT) and IFN-α. We found that EBV-positive lymphoma cells derived from these patients cultured with AZT express CD95 and undergo apoptosis. AZT-mediated apoptosis was caspase dependent and occurred despite Fas receptor blockade. In contrast, EBV-negative lymphomas were resistant to AZT-induced apoptosis, as were EBV-positive lymphomas that expressed high levels of bcl-2. Primary effusion lymphoma (PEL) cell lines infected with human herpesvirus type 8 required IFN-α to potentiate AZT-induced apoptosis. IFN-α did not up-regulate CD95 in BL or PEL but did induce expression of the death receptor ligand, CD95 ligand. AZT-sensitive lymphomas also accumulated significantly higher intracellular AZT monophosphate than did resistant lymphomas. Our data demonstrated distinct apoptotic responses to AZT and IFN-α in herpesvirus-associated lymphomas. EBV-positive BL cells that expressed low BCL-2 levels were sensitive to AZT alone; PEL cells required the addition of IFN-α to enhance apoptosis, and EBV-negative lymphomas were insensitive to both agents. AZT-sensitive BL cells transfected with BCL-2 became resistant. Susceptibility to antivirus-mediated apoptosis may be exploited to improve the therapy of certain herpesvirus-associated lymphomas.

INTRODUCTION

Lymphomas that occur in immunocompromised individuals are frequently associated with herpesviruses (1). AIDS NHLs[4] account for a large percentage of these cases in the United States. Approximately 30% of AIDS NHLs are categorized as Burkitt's type, and most of the rest are classified as diffuse or IBLs and primary central nervous system lymphomas (2, 3). A recently identified subtype of AIDS NHL is PEL associated with HHV-8 (4). AIDS BL commonly occurs in the setting of less severe immunosuppression than IBL, PEL, or primary cell nervous system lymphoma but grows more rapidly, resulting in severe metabolic abnormalities related to tumor lysis syndrome.

These categories of AIDS lymphoma are associated with certain molecular profiles. EBV is detected in BL in about 30% of cases and in the majority of large cell lymphomas and immunoblastic lymphomas. BL typically contains reciprocal translocations that juxtapose the c-*myc* gene near an immunoglobulin promoter. Large cell diffuse or immunoblastic lymphoma contains c-*myc* rearrangements less frequently but often carries mutations in BCL-6. BCL-2 overexpression is also more common in AIDS-related large cell types than BL (5–9). PEL usually presents as a malignant effusion without an apparent tumor mass in HIV-infected patients (4). These cellular and molecular characteristics, along with the different epidemiological profiles of AIDS NHLs, indicate that these tumors are biologically distinct.

This study of AIDS NHL primary patient tumor isolates, cell lines established from these patients, and published cell lines demonstrates that EBV-positive lymphomas and HHV-8-positive PEL cell lines are susceptible to AZT or AZT/IFN-α-mediated apoptosis. Apoptosis was associated with up-regulation of death receptor or death receptor ligands and activation of caspases. In contrast, AIDS B-cell lymphomas that expressed high levels of BCL-2 and EBV-negative lymphomas were resistant to AZT or AZT and IFN-α. These data indicate that AZT and IFN-α-mediated apoptosis may be specific for certain herpes virus-associated NHLs.

MATERIALS AND METHODS

Clinical Samples and Establishment of Primary Cell Lines. This study was performed under the guidelines of the University of Miami Institutional Review Board, where there is an ongoing protocol for AZT and IFN-α induction therapy for AIDS-related BL and PEL. All lymphomas studied were classified by independent reviews from two hematopathologists. Lymphomas classified as Burkitt's met criteria for small noncleaved cell lymphomas, Burkitt's type, according to the Working Formulation (10). Primary tumor isolates, cell lines established from AIDS NHL patients, and lines obtained from the American Type Tissue Culture Collection (Bethesda, MD) were used for this study.

Primary Patient Isolates. BL-3 was derived from a patient with AIDS-related, EBV-positive L3 (Burkitt's type) leukemia who responded to treatment with AZT and IFN-α (11). Flow cytometry of PBMCs demonstrated that 90% of circulating mononuclear cells were CD19 positive. These cells were positive by PCR for EBV. We were unable to establish BL-3 as a cell line, and cytogenetics were not performed.

BL-5 was derived from a diagnostic axillary node biopsy of a patient with AIDS-related BL who responded to AZT and IFN-α therapy. Cells from residual tissue of this specimen were cultured and after ~40 passages are now an established line. The BL-5 line is EBV positive (by PCR) and carries the most common c-*myc* 8;14 translocation.

SM-1 is a line established from a lymphomatous pericardial effusion from an AIDS patient. Flow cytometry demonstrated that 90% of these cells are CD19 positive. SM-1 has been established in culture for over 5 months and 30 passages. SM-1 is EBV positive and HHV-8 negative (by PCR) and carries an 8;14 translocation.

IBL-3 is a primary tumor cell culture of a malignant lymphomatous effusion derived from a patient unresponsive to AZT and IFN-α. The tumor was histopathologically classified as an IBL. It was negative for c-*myc* expression by Western blot. PCR analysis of the tumor was positive for EBV and negative for HHV-8. The tumor was not established as a continuous line.

IBL-4 was derived from a diagnostic thoracentesis performed on a patient unresponsive to AZT and IFN-α with AIDS-related IBL. The tumor has been established as a cell line (40 passages over 6 months). IBL-4 is CD19 positive,

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[4] The abbreviations used are: AIDS NHL, AIDS related non-Hodgkin's lymphoma; PEL, primary effusion lymphoma; HHV, human herpesvirus; AZT, azidothymidine; BL, Burkitt's lymphoma; IBL, immunoblastic large cell lymphoma; PBMC, peripheral blood mononuclear cell; CD95L, CD95 ligand; LDH, lactate dehydrogenase; PARP, poly(ADP-ribose) polymerase; LMP, latent membrane protein.

contains a balanced 2;8 translocation, and is EBV positive and HHV-8 negative by PCR.

BL-6 is a primary tumor cell culture obtained from a HIV patient with EBV-negative BL with circulating L3 lymphoblasts. Flow cytometry of the primary tumor specimen demonstrated that 90% of cells were CD19 positive. This patient was unresponsive to AZT and IFN-α. Cytogenetic analysis of the primary tumor cells was not done, and we were unable to establish a cell line.

Established Cell Lines. P3HR-1, Namalwa, and Daudi are EBV-positive lymphoma lines established from patients with endemic BL, and all contain the c-*myc* translocation. BJAB, Ramos, and D6 are EBV-negative B-cell lymphoma lines derived from HIV-negative patients. H9, a T-cell line, and B95-8, a marmocet line infected with EBV, were also used for our experiments. BCBL-1, BC-1, and BC-3 are lines developed from PEL patients. BCBL-1 and BC-3 are infected with HHV-8; BC-1 also contains EBV (12). These lines were obtained from the American Type Culture Collection.

All tumor cells and lines were maintained in culture with 10% heat-inactivated FCS in Iscove's modified Dulbecco's media (Life Technologies, Inc., Grand Island, NY).

Reagents and Antibodies. The caspase protease inhibitors, BocD-fmk and zVAD-fmk, were purchased from Enzyme Systems Products (Dublin, CA) and prepared in anhydrous DMSO. For apoptosis inhibition assays, caspase inhibitors were added to cells prior to drug treatment or addition of CD95 agonist antibody (clone DX-2) from PharMingen (San Diego, CA).

Immunostaining of cells for CD95 expression was performed with mouse anti-human CD95 FITC or CD95 PE (clone DX2) from PharMingen. Anti-CD95 blocking antibody (clone ZB4) was purchased from Coulter Immunotech (Miami, FL).

Cytotoxicity Assays. Target cell lysis was assessed by ^{51}Cr release assay. Briefly, 1×10^6 target cells were incubated with 200 μCi of Na_2-$^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 1 h, washed, and plated into round-bottomed 96-well tissue plates at 1×10^4 target cells/well in triplicate. The target cells were incubated with effector cells at the indicated E:T ratios in a total volume of 200 μl of media for 4 h at 37°C in a CO_2 incubator. In assays of cytotoxicity against CD95-expressing targets, we used as effector cells the murine lymphoma line EL-4, stably transfected with CD95 ligand (CD95L; EL4-mFL). Cytotoxicity assay supernatant (100 μl) was gamma counted, and the percentage of specific lysis was calculated by subtraction of spontaneous lysis from target cell lysis divided by the difference between complete and spontaneous lysis (target cells incubated with media alone). Complete target cell ^{51}Cr release was induced by total cell lysis with HCl. Cytotoxicity assays were performed in triplicate per experiment and repeated at least twice.

Transfection of BL-5 with BCL-2. BCL-2 transfectants were prepared as described previously (13). BL-5 cells were transfected by electroporation with the pSFFV-NEO plasmid containing BCL-2a in transcriptional orientation. As a control, transfection was also performed with the pSFFV-NEO plasmid without an insert. Cells were cultured in medium containing G418 (1 mg/ml). Transfectants with acquisition of neomycin resistance were selected. Single-cell clones were generated by limiting dilution. The expression of BCL-2 in selected single cell clones was confirmed by Western blot. The selected clone, BL-5-BCL-2, expressed similar baseline levels of CD95 compared with BL-5 cells and BL-5-neo controls.

Flow Cytometry. Antibody staining was performed on one million cells for each conjugated primary antibody. FITC or PE-conjugated mouse antihuman CD95 antibody (DX-2; PharMingen) or isotype control antibodies were used according to the manufacturer's recommendations, at a concentration of 5 μg/ml, in PBS/0.5% albumin/0.1% sodium azide (PBA) in 50 μl. Stained cells were placed on ice for 30 min, washed twice, and then immediately analyzed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA). Analysis of CD95 expression levels was performed on live cells gated by forward and side scatter.

Apoptosis was assayed with propidium iodide/annexin V staining. For propidium iodide/annexin V-FITC staining of apoptosis, cells were washed with annexin V FACS buffer (HBSS buffer with calcium, magnesium, sodium azide, and 0.5% BSA) and incubated on ice with a 1:30 dilution of annexin V-FITC (Caltag Laboratories, Burlingame, CA) for 30 min and washed in FACS buffer. Prior to FACS analysis, propidium iodide at 5 μg/ml (Sigma Chemical Co., St. Louis, MO) was added to the cells and gently mixed. Stained cells were immediately analyzed by FACS.

Semiquantitative Reverse Transcription-PCR and ELISA for CD95L. Total RNA was prepared according to the manufacturer's specifications (RNAeasy; Qiagen, Valencia, CA) from aliquots of 10^6 cells. One μg of total RNA was resuspended in 12.5 μl of RNase free water, and 1 μl of oligo(dT) was added, and the mixture was heated for 2 min at 90°C. cDNA was synthesized in a reaction mixture (6.5 μl) containing 1 μl of Moloney murine leukemic virus reverse transcriptase (200 u/μl), 1 μl of deoxynucleotide triphosphate mixture (10 mM each), 0.5 μl of RNase inhibitor (40 u/μl), 25 mM Tris (pH 8.3), 37 mM KCl, 1.5 mM MgCl, and 5 mM DTT. The tubes were incubated for 1 h, and the reaction was stopped by incubation at 94°C for 5 min. PCR was performed according to methods published previously (14), on cDNA using 0.25 μM concentrations of primer pairs specific for CD95L

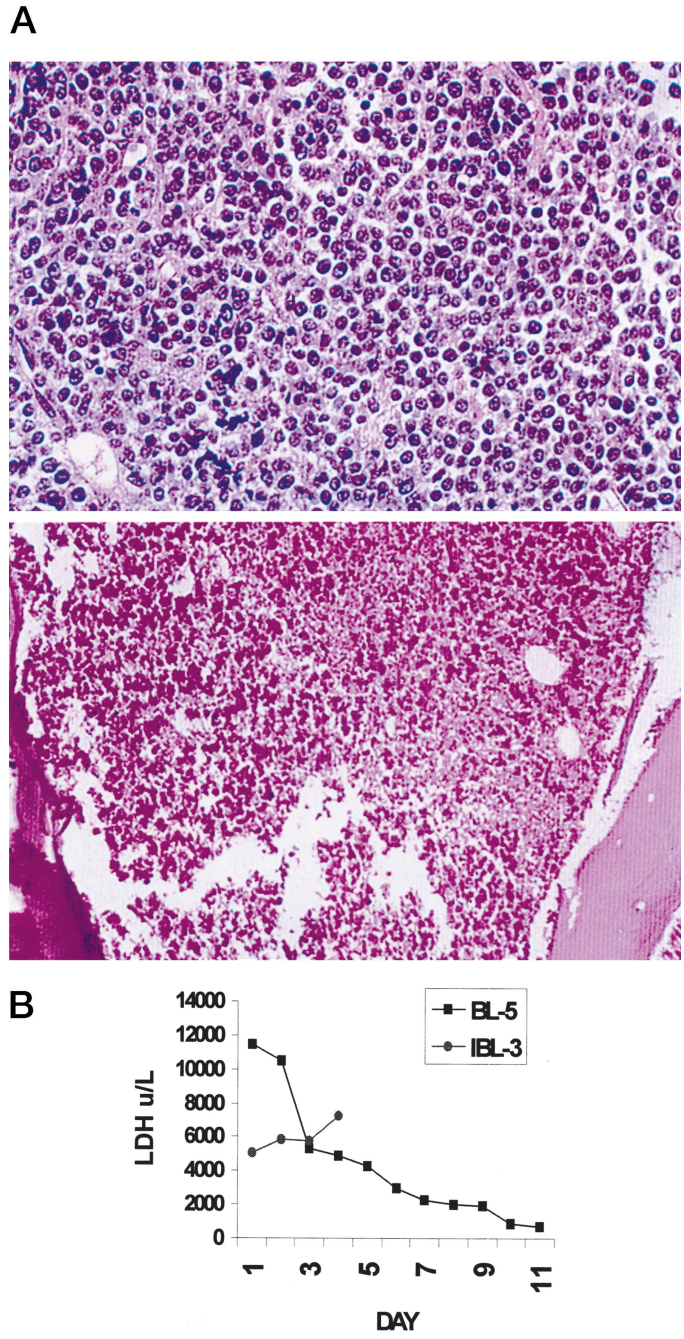

Fig. 1. Antiviral therapy results in decreased tumor burden in HIV-related BL. *A*, Wright Giemsa stain of iliac crest biopsies performed on day 0 (*upper panel*) and day 15 (*lower panel*) in a patient with HIV-related, EBV-positive BL. *B*, tumor burden and tumor lysis were monitored by daily measurement of serum LDH after AZT and IFN-α antiviral therapy. ■, LDH concentration in the serum of a patient with BL (BL-5); ●, LDH levels from a patient with IBL (IBL-3).

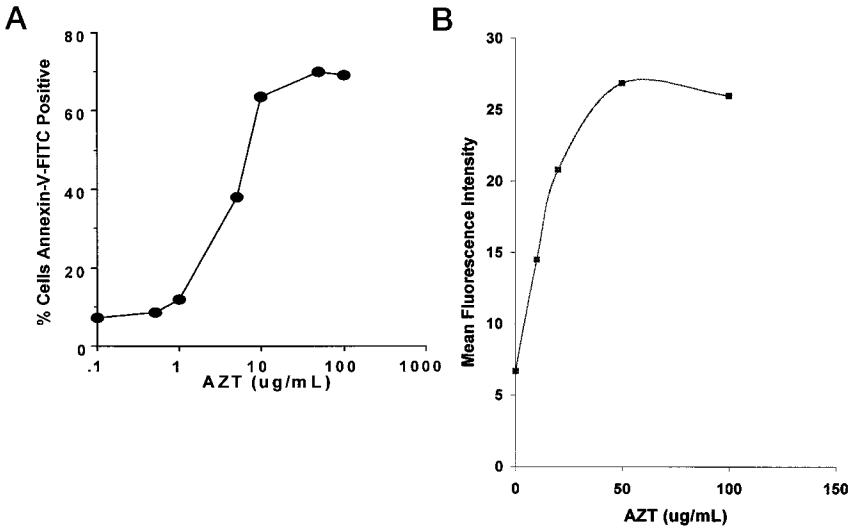

Fig. 2. AZT induces CD-95 expression and apoptosis in BL-5 but not IBL-3 and IBL-4. *A*, BL-5 cells were treated for 48 h with titrating doses of AZT and analyzed for apoptosis by propidium iodide/annexin flow cytometry. *B*, dose-response of CD95 expression by AZT. BL-5 was treated with AZT for 40 h at the indicated concentrations, and CD95 expression was monitored by flow cytometry. The data are representative of three independent experiments. *C*, cells from BL-5 (*left panel*) cultured for 48 h in the presence (*solid line*) or absence (*dashed line*) of AZT 5 μg/ml and analyzed for CD95 expression by flow cytometry. *Dotted line*, isotype control (mouse IgG-1). Cells from IBL-3 (*center panel*) and IBL-4 (*right panel*) were cultured in the presence (*solid line*) or absence (*dashed line*) of AZT 100 μg/ml, and CD95 expression was analyzed by flow cytometry [*dotted line*, isotype control (mouse IgG-1) in BL-5 and IBL-4].

(5′ primer, 5′-CGCCACCACTGCCTCCACTA-3′; 3′ primer, 5′-CTTCCCCTCCATCATCACCA-3′) containing 0.25 mM of each deoxynucleotide triphosphate, 10 mM Tris (pH 9.0), 5 mM KCl 0.1%, 1.5 mM $MgCl_2$ and 0.2 unit Taq DNA for 35 cycles (denaturation for 1.5 min at 94°C, annealing for 1.5 min at 60°C, and extension for 1 min at 72°C). PCR products were equalized using amplified product of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase as a standard. ELISA for CD95L was performed on control cells and cells treated with IFN-α (1000 units/ml) and cultured for 48 h using the MBL Biotechnology ELISA kit (Watertown, MA).

PCR Screening for EBV and HHV-8. DNA was tested for the presence of the EBV BMLF1 as described previously (15). The two primers are 5′-CACCACCTTGTTTTGACGGG-3′ and 5′-GTCAACCAACAAGGACACAT-3′, which amplify a fragment of 304 bp. The amplified products were then analyzed on a 1% agarose gel. Amplification of the KS 330_{233} bp fragment on DNA extracted from tumor samples was performed on malignant lymphomatous effusions (suspected PEL), using HHV-8-specific primers (5′-AGCCGAAAGGATTCCACCATT-3′ and 5′-TCCGTGTTGTCTACGTCCAGA-3′) and published conditions (4), and analyzed on a 1% agarose ethidium bromide gel.

Western Blot Analysis. Cell lysates prepared from primary tumor cells (3×10^6) in RIPA buffer were resolved on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose and after blocking with nonfat dry milk, immunoblot analyses were performed using a hamster monoclonal IgG antibody against human BCL-2 (PharMingen), followed by a mouse antihamster IgG secondary antibody (PharMingen). This was followed by a horse radish peroxidase conjugate sheep antimouse IgG at 1:2000 dilution (ECL; Amersham, Arlington Heights, IL). For c-*myc*, caspase-3, and PARP immunoblots, we used PharMingen antibodies followed by the ECL detection system (Amersham). LMP-1 immunoblotting was performed with a Dako (Glostrup, Denmark) antibody cocktail that detects LMP-1 (epitopes CS 1–4). When using this antibody, typically 2 bands appear in LMP-1-positive Western blots (16).

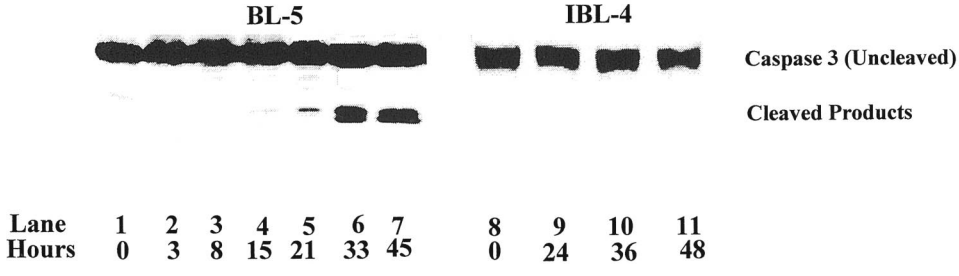

Fig. 3. AZT induces apoptosis in BL. AZT activates caspase 3. BL-5 and IBL-4 cells were treated with AZT 5 μg/ml over a 2-day time course. *Lanes 1–7*, time-dependent caspase 3 activation in BL-5 cells. *Lanes 8–11*, only uncleaved caspase 3 in AZT-treated IBL-4 cells. AZT induced maximal formation of the activated cleaved caspase 3 by 30–40 h in BL-5.

AZT Phosphorylation Assay. Intracellular levels of phosphorylated AZT were measured using published methods (17). Cells were plated out in 24-well plates at 10^6 cells/ml/well and incubated with 5 μg/ml [^{3}H]AZT. After 24 h of incubation, cells were harvested and washed with PBS twice. Cell pellets were extracted with cold 65% methanol on ice for 30 min and spun at 1500 rpm for 5 min. The supernatants were collected and applied to high-performance liquid chromatography for measurements of the monophosphate, diphosphate, and triphosphate forms of AZT.

RESULTS

Clinical Activity of AZT and IFN-α in AIDS NHL. We reported previously that AIDS BL patients responded to parenteral AZT and IFN-α therapy with gradual regression of lymphoma and tumor lysis over a 2-week period. Response was demonstrated by regression of lymphadenopathy, splenomegaly, and leukemia (data not shown). In contrast, AIDS IBL patients worsened on AZT and IFN-α therapy and required conventional chemotherapy within 4–5 days (11). Fig. 1*A* demonstrates the clearance of lymphoma involving the bone marrow in a patient with EBV-positive BL after 2 weeks of parenteral AZT and IFN-α therapy. To test whether selective antiviral-induced apoptosis was the mechanism of this disparate response, we isolated primary tumor cells prior to AZT and IFN-α therapy from a BL patient (BL-5) and from a patient with IBL (IBL-3). Clinically, these patients displayed opposite responses to AZT and IFN-α. LDH, a marker of tumor burden, declined in patient BL-5 during AZT and IFN-α treatment, coinciding with marked regression of tumor burden (Fig. 1*B*). IBL-3 did not respond and clinically worsened over the first 4 days of therapy. Because we typically had seen objective tumor regression within this time period in responsive patients, AZT and IFN-α therapy was stopped for ethical reasons.

***In Vitro* Induction of Apoptosis and CD95 Expression Correlates with Clinical Response.** We investigated whether the clinical response to AZT and IFN-α correlated with the induction of apoptosis in cultured tumor cells. BL-5 and IBL-3 cells were cultured for 48 h in AZT and apoptosis was measured by annexin V flow cytometry. AZT induced apoptosis in a time- and dose-dependent manner in BL-5 (Fig. 2*A*). AZT had no effect on IBL-3 cells, and IFN-α did not increase apoptosis in BL-5, IBL-3, or IBL-4 (data not shown).

Because previous work suggested that conventional chemotherapeutic agents induce apoptosis in sensitive cells through CD95 (also known as Fas or APO-1) signaling (18), we investigated whether AZT up-regulates this proapoptotic molecule on BL-5. CD95 expression, as measured by flow cytometry, was induced on BL-5 cells in a dose-dependent manner (Fig. 2*B*). CD95 was not induced on IBL-3 or IBL-4 cells by even higher doses of AZT (100 μg/ml; Fig. 2*C*). CD95 induced by AZT also was competent to signal apoptosis by agonistic CD95 cross-linked antibody (clone DX-2; data not shown).

Induction of Apoptosis by AZT Is Caspase Dependent. Cleavage of caspase-3 and other protein substrates are events that occur in a cell committed to apoptosis (19, 20). AZT, in a time-dependent manner, induced cleavage of caspase-3 into the active protease in BL-5 cells. In contrast, caspase 3 cleavage was not detected in IBL-4 cells treated with AZT (Fig. 3). AZT treatment of BL-5 also was associated with cleavage of PARP, as detected by Western blotting. PARP cleavage in BL-5 was blocked by the broad spectrum caspase inhibitor Boc-D-fmk (data not shown).

Induction of Apoptosis in HHV-8-positive PEL by AZT and IFN-α. We studied the effects of AZT on the HHV-8-positive PEL cell line, BCBL-1. Although AZT also induced CD95 on BCBL-1, apoptosis was much less than that observed in BL-5. We therefore studied whether the CD95 induced by AZT was functional by using EL-4 murine lymphoma cells transfected with CD95 ligand (EL4-mFL) as effector cells in a cellular cytotoxicity assay. EL4-mFL transfectants induced significant specific lysis of BCBL-1 cells pretreated with AZT compared with nontreated BCBL-1 target cells (Fig. 4*A*). This cellular cytotoxicity was specific for the CD95 pathway

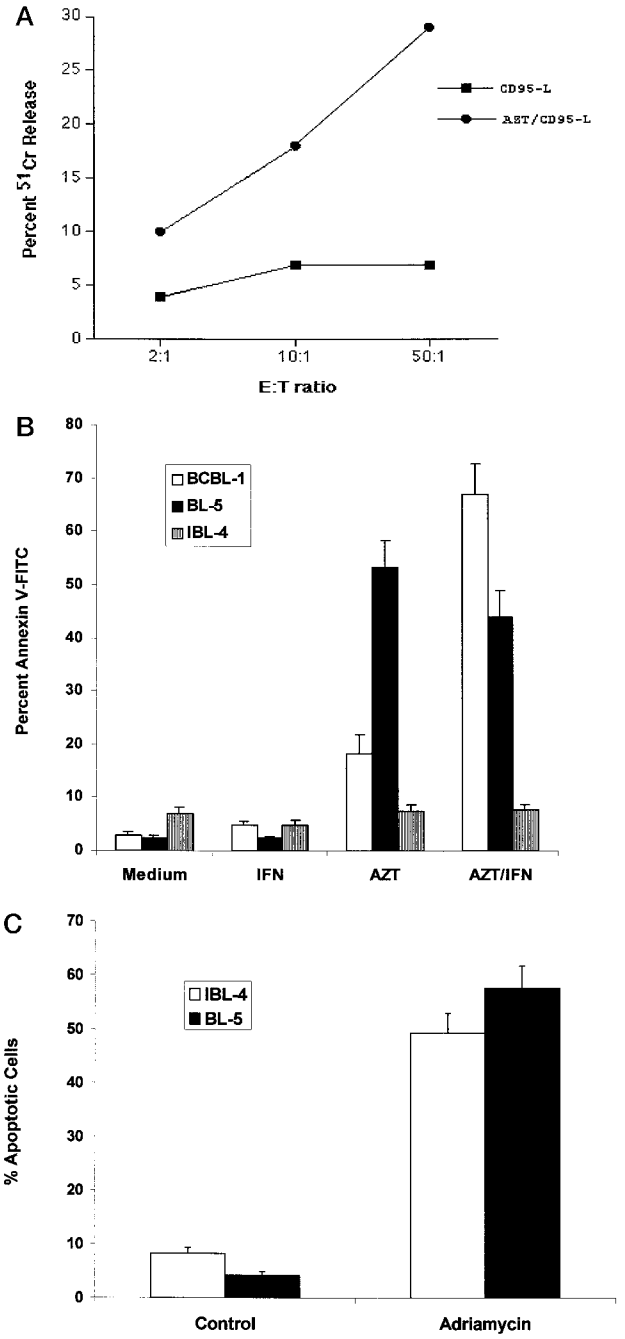

Fig. 4. BCBL-1 undergoes apoptosis in response to AZT and IFN α. *A*, induction of CD95L-mediated cytotoxicity is potentiated by AZT. BCBL-1 cells were cultured in the presence (●) or absence (■) of AZT (10 μg/ml) for 48 h. Cells were then ^{51}Cr-labeled and cocultured with EL-4 cells stably transfected with murine CD95L (EL4-mfl) for 4 h. Specific ^{51}Cr release was measured by determining the cpm released into the culture supernatant. The data are presented as the means of triplicate samples and represent two independent experiments. *B*, IFN-α potentiates apoptosis in AZT-treated BCBL-1. BCBL-1, BL-5, and IBL-4 cells were cultured for 72 h in medium, IFN-α (1000 units/ml), AZT (5 μg/ml), or AZT (5 μg/ml) and IFN α (1000 u/ml) together and assayed for apoptosis by annexin V-FITC staining and FACS analysis. *Bars*, SD. *C*, IBL-4 and BL-5 are sensitive to Adriamycin. IBL-4 and BL-5 cells were cultured in medium or Adriamycin 0.5 μg/ml for 48 h, and apoptosis was measured by annexin V staining. Data are presented as the percentage of annexin-positive cells; *bars*, SD.

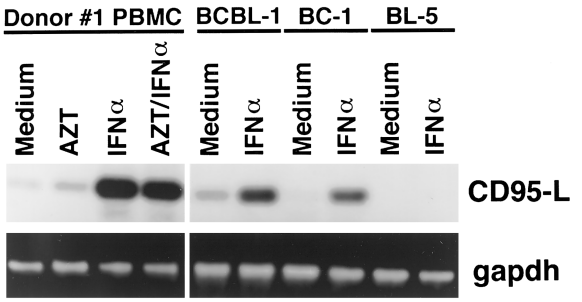

Fig. 5. IFN-α induces CD95L. Normal PBMCs, BCBL-1, BC-1, and BL-5 cells were treated as indicated in the figure for 5 h. AZT treatment was performed at 100 μg/ml and/or IFN-α 1000 units/ml. Cells were then lysed, and total RNA was extracted and hybridized with a DNA probe specific for CD95L. *gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

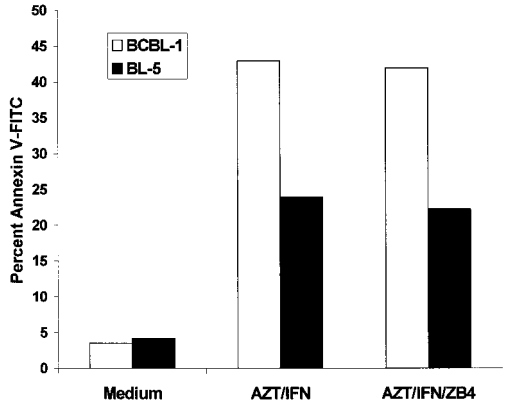

Fig. 6. CD95 receptor blockade does not inhibit AZT and IFN-α-mediated apoptosis. BCBL-1 and BL-5 cells were treated for 48 h with AZT (5 μg/ml) and IFN-α (1000 u/ml) in the presence or absence of anti-CD95 blocking antibody ZB4. Apoptosis was measured by FACS analysis of annexin V-FITC staining.

because the EL4 cells were transfected with only CD95L. In addition, EL4 mock transfectants did not lyse BCBL-1, and EL4-mFL cytotoxicity against AZT treated BCBL-1 was blocked by CD95 blocking antibody (clone ZB4; data not shown). This demonstrated that AZT also induces functional CD95 on BCBL-1.

Because IFN-α is included in our antiviral chemotherapy clinical protocol, we characterized the response of BCBL-1, BL-5, and the IBL-4 lymphoma line to treatment with AZT, IFN-α, or both agents together. BL-5 was sensitive to AZT-induced apoptosis, and IFN-α had no additive effect. BCBL-1 was relatively resistant to apoptosis induced by AZT alone but underwent significantly increased apoptosis upon treatment with both AZT and IFN-α. IBL-4 cells were resistant to either agent alone or in combination (Fig. 4*B*). A similar synergy between AZT and IFN-α-induced apoptosis was observed in another HHV-8-positive PEL cell line, BC-3 (data not shown). Resistance to AZT in IBL-4 cells did not correlate with resistance to Adriamycin. Both BL-5 and IBL-4 were quite sensitive to apoptosis induced by Adriamycin (Fig. 4*C*).

IFN-α Induces CD95L Expression. Although AZT alone induced functional CD95 expression in BCBL-1, apoptosis was markedly enhanced by the addition of IFN-α. We therefore studied whether IFN-α could induce CD95L. Primary lymphoma BL cells (BL-5), PEL lymphoma cell lines (BCBL-1 and BC-1), and PBMCs from a normal donor cultured in the presence of IFN-α were assayed for CD95L expression using semiquantitative reverse transcription-PCR. CD95L transcripts were significantly induced in the PEL cell lines BCBL-1 and BC-1 but not in BL-5. CD95L was also strongly induced in PBMCs by IFN-α (Fig. 5). IFN-α treated PBMCs also expressed higher levels of CD95L then control PBMCs, as measured by ELISA (data not shown).

Fas Blockade Does Not Inhibit AZT and IFN α-mediated Apoptosis. Although in BCBL-1, CD95 was induced by AZT and CD95L by IFN-α, the cytopathic effect of this combination occurred in both BL-5 and BCBL-1, despite CD95 receptor blockade by the anti-CD95 antibody, Zb4 (Fig. 6). Zb-4 did block EL-4mFas-L transfectants directed against Fas-positive BCBL-1 cells as well as agonist

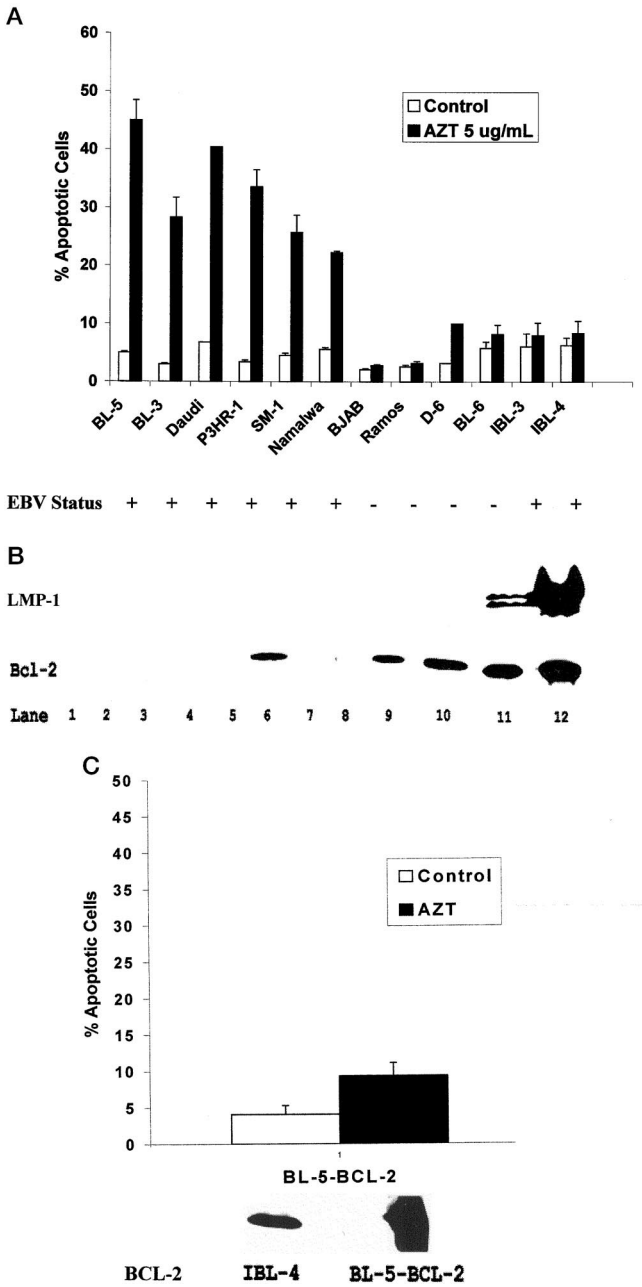

Fig. 7. AZT selectively induces apoptosis in EBV-positive, BCL-2-negative cells. *A*, EBV-positive Burkitt's cells (BL-5, BL-3, SM-1, Daudi, Namalwa, and P3HR-1). EBV-negative lymphomas (BJAB, Ramos, D6, and BL-6) and immunoblastic lymphoma cells (IBL-3 and IBL-4) were treated for 48 h in medium or AZT 5 μg/ml. Apoptosis was measured by annexin V flow cytometry. Data are presented as the percentage of apoptotic cells in each fraction. *B*, baseline expression levels of BCL-2 were determined by Western blot in each *lane*. *Lane 1*, BL-5; *Lane 2*, BL-3; *Lane 3*, Daudi; *Lane 4*, P3HR-1; *Lane 5*, SM-1; *Lane 6*, Namalwa; *Lane 7*, BJAB; *Lane 8*, Ramos; *Lane 9*, D-6; *Lane 10*, BL-6; *Lane 11*, IBL-3; *Lane 12*, IBL-4. LMP-1 Western blots were performed on all EBV-positive lines. *C*, BL-5 cells transfected with BCL-2 (*BL-5-BCL-2*) were treated for 48 h in medium or AZT 5 μg/ml, and apoptosis was measured by annexin V staining. *Bottom panel*, BCL-2 expression levels for IBL-4 and BL-5-BCL-2.

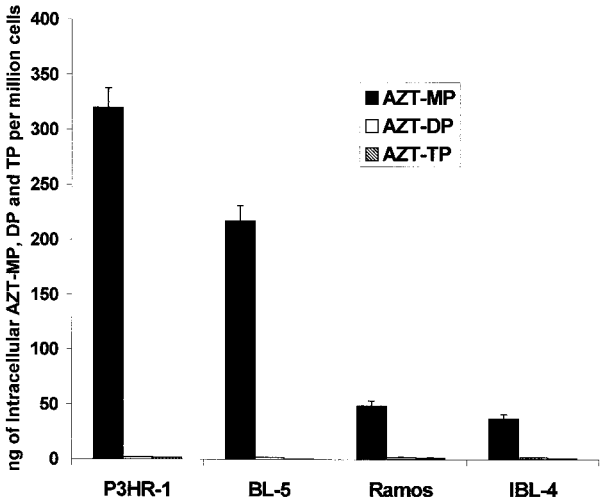

Fig. 8. AZT-induced apoptosis correlated with intracellular AZT-MP levels. AZT-sensitive (*P3HR-1* and *BL-5*) and resistant (*Ramos* and *IBL-4*) cell lines were cultured in the presence of tritiated AZT for 24 h. Intracellular levels of AZT-MP, DP, or TP were determined by high-performance liquid chromatography. Data are presented as nanograms of each form of phosphorylated AZT per million cells. *Bars*, SD.

anti-Fas antibody CH-11 directed against H-9 cells (data not shown). This indicates that the mechanism of apoptosis activated by these agents is more complex than induction of a CD95/CD95L interaction, although if multiple apoptosis pathways are activated by AZT and IFN-α, blockade of one pathway may show no inhibition in overall cytotoxicity (21).

Differential Apoptosis of NHL Subtypes Induced by AZT. To determine whether apoptosis induced by AZT targets certain types of tumor cells, we tested a panel of B-cell lymphomas including lines derived from AIDS patients and published lines. All AZT-sensitive primary cells and lines were EBV positive (BL-5, BL-3, SM-1, Daudi, P3HR-1, and Namalwa). Resistance to AZT correlated with the level of BCL-2 detected by Western blot in EBV-positive lines (IBL-3 and IBL-4) or the absence of EBV (BJAB, Ramos, D6, and BL-6). Both IBL-3 and IBL-4 also expressed high levels of LMP-1 protein (Fig. 7, *A* and *B*). An EBV-negative T-cell line, H-9, and B95–8, an EBV-infected line that expresses BCL-2 and LMP-1, were also resistant to AZT (data not shown). To study whether BCL-2 expression could change an AZT-sensitive line to a resistant phenotype, we transfected BL-5 with BCL-2. As demonstrated in Fig. 7*C*, BL-5-BCL-2 cells were much more resistant to AZT-mediated apoptosis.

Intracellular AZT-MP Levels Correlate with Sensitivity to AZT-mediated Apoptosis. Intracellular AZT is phosphorylated to mono-, di-, and triphosphate. The triphosphate (AZT-TP) competes with TTP (TTP-TP) for utilization by HIV reverse transcriptase, thereby inhibiting viral replication. However, AZT monophosphate (AZT-MP) is believed to be a cytotoxic intermediate (22, 23). We therefore investigated whether the cytopathic effect of AZT in cells correlated with the intracellular levels of AZT-MP. We cultured the AZT-sensitive (BL-5 and P3HR-1) and -resistant (IBL-4 and Ramos) lines in the presence of tritiated AZT 5 μg/ml for 24 h and measured the relative levels of the phosphorylated forms of AZT by high-performance liquid chromatography. A markedly higher level of AZT-MP was detected in the sensitive P3HR-1 and BL-5 lines than in the resistant IBL-4 and Ramos lines (Fig. 8). This suggests that AZT-MP may preferentially accumulate in certain EBV-positive lymphoma cells sensitive to AZT-mediated apoptosis.

DISCUSSION

AIDS NHL grows so rapidly that patients often present with moderate to severe tumor lysis syndrome. Although apoptotic death of tumor cell lines treated with chemotherapeutic agents has been demonstrated *in vitro* (18), in the clinical setting cytotoxic chemotherapy often exacerbates necrotic cell death and tumor lysis syndrome, resulting in further release of proteolytic enzymes and tissue damage (24). This complication is particularly common in aggressive lymphomas; therefore, a therapy that induces apoptosis of these tumors *in vivo* would be of great value. The gradual regression of lymphoma and resolution of tumor lysis syndrome of our AIDS BL patients treated with AZT/IFN-α suggested to us that their clinical response was through an apoptotic rather than a necrotic type of cellular death.

We found that EBV-positive BL cells from these AIDS patients responded *in vitro* to AZT by undergoing apoptosis. AZT-sensitive cells underwent apoptotic membrane changes, cleaved their PARP, and died through a caspase-dependent pathway. AZT also induced elevated expression of CD95 competent to signal apoptosis when triggered by an agonistic antibody. Thus, one mechanism of the clinical response observed in these patients may be through up-regulation of CD95 that is subsequently signaled by circulating activated tumor-specific CTLs expressing CD95L, resulting in apoptotic death. Surprisingly, only EBV-positive lymphomas were sensitive to AZT-mediated apoptosis. EBV-negative Burkitt's lines (BJAB, D6, and Ramos), an AIDS-related EBV-negative BL (BL-6), and another T-cell line (H9) were resistant to AZT. It is possible that EBV plays a role in susceptibility to AZT.

Although we did not treat any PEL patients with AZT and IFN-α, AZT induced apoptosis in HHV-8-positive PEL cell lines that was markedly potentiated by IFN-α. The induction of CD95L by IFN-α in these cells may partially explain why these lymphoma lines were sensitive to the combination of these agents. If CD95 is up-regulated by AZT in tumor cells *in vivo*, IFN-α may increase the expression of Fas-L in cytotoxic cells. IFN-α is an endogenous cytokine, and one of its functions may be to up-regulate certain death receptor ligands such as CD95L in response to stimuli such as viral infections. AZT- and IFN-α-induced apoptosis occurred *in vitro* despite the presence of CD95 blocking antibodies. This suggests that CD95 up-regulation may be a marker for lymphomas that are responsive to AZT/IFN-α therapy but are not the sole pathway of apoptosis. However, if circulating BL cells express CD95 after parenteral AZT therapy, clearance by IFN-α-stimulated CD95L-bearing cells may be enhanced.

The EBV-positive AIDS lymphomas that overexpressed BCL-2 were resistant to AZT-mediated apoptosis. Overexpression of BCL-2 in a sensitive lymphoma line also rendered it resistant to AZT. Because BCL-2 may be expressed at high levels in AIDS NHL, this therapy may not be effective in EBV-positive lymphomas that overexpress this antiapoptotic protein. A recent pathological review of AIDS NHLs indicates that BCL-2 expression is less frequent in AIDS BL (9); however; most AIDS BLs are also EBV negative (8). In contrast, endemic BL is virtually always EBV positive (25, 26); therefore AZT or AZT and IFN-α may be quite active in this lymphoma subtype. Our data also support this because AZT was markedly proapoptotic in lymphoma lines derived from endemic BL.

AZT, originally developed as a chemotherapeutic agent, has activity in adult T-cell leukemia (27) but has been recently used almost exclusively as an antiretroviral agent. Its adverse clinical effects are thought to be related to mitochondrial toxicity (28). Our data demonstrated a correlation between susceptibility to AZT and intracellular accumulation of AZT-MP. The factors responsible for the marked increase in AZT-MP in EBV-positive BLs, P3HR-1, and BL-5, are

unclear but could involve phosphorylation by herpesvirus kinases. It would be necessary to study AZT-MP levels in tumor cells taken from patients on therapy, as well as more cell lines, to determine whether this occurs *in vivo* or is exclusively an *in vitro* phenomenon. If apoptosis was triggered after intracellular accumulation of AZT-MP, this would explain why Fas receptor blockade did not inhibit apoptosis *in vitro*.

In summary, our data indicate there are at least three different responses to the effects of AZT or AZT and IFN-α in patients with high-grade B-cell lymphomas: (*a*) EBV-positive lymphomas that express low levels of BCL-2, up-regulated CD95, and underwent apoptosis upon exposure to AZT. Patients with these types of tumor may benefit from these agents, perhaps given in conjunction with or prior to standard chemotherapy; (*b*) EBV-positive lymphomas that express high levels of BCL-2 (and LMP-1) or EBV-negative lymphomas were resistant to AZT (or AZT and IFN-α). Patients with these types of tumors might not benefit from these agents; (*c*) HHV-8-positive PEL cells (BCBL-1 and BL-3) were minimally sensitive to AZT and required IFN-α for a significant apoptotic effect. Because we did not treat PEL patients with these agents, we can say little of their potential efficacy in this disease. Although these findings are based on a limited number of samples, the data suggest that some herpesvirus-associated lymphomas may be responsive to AZT and IFN-α. Herpesvirus-associated lymphomas are also seen in a variety of clinical scenarios, including hereditary immunodeficiencies and iatrogenic immunosuppression after organ transplantation (29, 30). To confirm these findings, more isolates need to be studied. However, if these observations are valid, they can be translated into clinical trials that use antivirals as part of a regimen. Anticancer agents that induce tumor apoptosis *in vivo* would also likely lessen patient morbidity from complications of tumor lysis caused by standard chemotherapy agents.

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