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Involvement of Glutathione as a Mechanism of Indirect Protection against Spontaneous Ex Vivo Apoptosis Associated with Bovine Leukemia Virus

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Viruses have developed strategies to counteract the apoptotic response of the infected host cells. Modulation of apoptosis is also thought to be a major component of viral persistence and progression to leukemia induced by retroviruses like human T-lymphotropic virus type 1 (HTLV-1) and bovine leukemia virus (BLV). Here, we analyzed the mechanism of ex vivo apoptosis occurring after isolation of peripheral blood mononuclear cells from BLV-infected sheep. We show that spontaneous apoptosis of ovine B lymphocytes requires at least in part a caspase 8-dependent pathway regardless of viral infection. Cell death is independent of cytotoxic response and does not involve the tumor necrosis factor alpha/NF-κB/nitric oxide synthase/cyclooxygenase pathway. In contrast, pharmaceutical depletion of reduced glutathione (namely, γ-glutamyl-L-cysteinyl-glycine [GSH]) by using ethacrynic acid or 1-pyrrolidinecarbodithioic acid specifically reverts inhibition of spontaneous apoptosis conferred indirectly by protective BLV-conditioned media; inversely, exogenously provided membrane-permeable GSH-monoethyl ester restores cell viability in B lymphocytes of BLV-infected sheep. Most importantly, intracellular GSH levels correlate with virus-associated protection against apoptosis but not with general inhibition of cell death induced by polyclonal activators, such as phorbol esters and ionomycin. Finally, inhibition of apoptosis does not correlate with the activities of GSH peroxidase and GSH reductase. In summary, our data fit into a model in which modulation of the glutathione system is a key event involved in indirect inhibition of apoptosis associated with BLV. These observations could have decisive effects during therapeutic treatment of δ-retroviral pathogenesis.

During evolution viruses have developed strategies, such as inhibition of humoral immunity, evasion of cytotoxic response, interferon (IFN) interference, repression of apoptosis, or modulation of cytokines and chemokines, permitting continuous persistence within the host (recently reviewed in reference 1). Viral infection indeed requires the production of proteins allowing completion of the replication cycle and/or stimulation of cell proliferation. One of these factors is the Tax protein expressed by complex oncoviruses (recently reclassified as δ-retroviruses) which comprise a series of pathogens infecting primates and ruminants (15, 52, 57). On the other hand, host cells express proteins, like p53, that counteract viral spread and oncogenic stimulation. The mechanisms of δ-retrovirus–host interplay have been a very active and disputed area of research, Tax either being proapoptotic (6, 7, 14, 17, 21, 24, 38) or acting as an inhibitor of cell death in other experimental systems (9, 22, 27, 29, 33, 35, 38, 48, 55). Together, these conflicting observations suggest that Tax might have different effects depending on the assay conditions, such as the levels of protein expression, the cell type specificity (fibroblast or lymphocyte), or the oncogenic status (primary or immortalized). Therefore, recent protocol developments have focused on primary cells directly isolated from infected hosts and briefly cultivated ex vivo (11, 12, 18, 19). The advantage of this approach is that the apoptotic process can be analyzed within the correct cell type in the context of a complete proviral genome and under conditions thought to closely reflect the natural situation occurring in vivo.

We thus aim to cast light onto the metabolic pathways involved in apoptosis associated with a member of the δ-retrovirus genus, called bovine leukemia virus (BLV), used as a model for the related human T-lymphotropic virus type 1 (HTLV-1). These two viruses infect lymphoid cells, possibly leading to hematological or neurological disorders (15, 52, 57): adult T-cell leukemia/lymphoma (ATL) or tropical spastic paraparesis/HTLV-associated myelopathy induced by HTLV-1 and bovine leukemia associated with BLV. Although these two viruses share a number of structural and functional homologies (i.e., similar genomic organization, random integration within the host chromosome, absence of cell-derived oncogene, and apparent lack of transcription in vivo), they exhibit marked differences in cell type specificities (CD4 or CD8 for HTLV-1 and B lymphocyte for BLV) and host targets (human and cattle, respectively). It has previously been demonstrated that BLV modulates spontaneous apoptosis initiated after short term cultivation (11, 12, 41). In fact, ovine cells expressing viral proteins ex vivo are completely spared from programmed death, and this inhibition also correlates with a concomitant

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reduction in the number of apoptotic B lymphocytes. Besides this direct effect, conditioned media from peripheral blood mononuclear cells from BLV-infected cows and sheep also indirectly decrease apoptosis (11, 12), suggesting that a protective factor might be present in these supernatants. In this report we characterize the metabolic pathways involved in this antiapoptotic process.

MATERIALS AND METHODS

Experimental animals. Sheep were kept under controlled conditions at the Veterinary and Agrochemical Research Centre (Machelen, Belgium). At regular time intervals, the total leukocyte counts were measured by using a Coulter counter ZN and the number of lymphocytes was estimated after morphological examination under the microscope. In parallel, the serum from each sheep was analyzed for BLV seropositivity by using immunodiffusion and enzyme-linked immunosorbent assay (ELISA) techniques (34). BLV-infected sheep were at different stages of the disease: nos. 104, 105, 282, 1095, and 2672 were asymptomatic, nos. 8, 245, 293, and 2675 were characterized by an inverted B-lymphocyte/T-lymphocyte ratio (prelymphocytic), and nos. 235 and 273 were leukemic (leukocytes above 10,000 cells per mm^3). All these sheep were infected with a wild-type proviral clone of BLV strain 344 (pBLV344 for nos. 235 and 273 and pBLVIX for nos. 8, 105, 282, 293, 1095, and 2672), except for no. 104 (49) and no. 245 (53), which were infected with a Tax mutant and a R3/G4 recombinant, respectively, and no. 2675, which was mutated in the gp51 envelope protein. Importantly, based on infectivity, viral spread, and pathogenesis, all these viruses behaved like the wild type in vivo (52 and unpublished results). As controls, uninfected animals (nos. 112, 114, 115, 117, 1097, and 2274) were housed under the same conditions.

Isolation of PBMCs and cell culture. Peripheral blood mononuclear cells (PBMCs) were isolated by Percoll gradient centrifugation as described previously (12). Briefly, venous blood was collected by jugular venipuncture and mixed with EDTA, which was used as an anticoagulant (1 ml of 7.5% EDTA per 25 ml of blood). PBMCs were then separated by Percoll density gradient centrifugation (Amersham Pharmacia Biotech) and washed (twice with phosphate-buffered saline [PBS]–0.075% EDTA and at least three times with PBS alone). After estimation of their viability by trypan blue dye exclusion, 4×10^6 cells were cultivated for 18 h at 37°C in a 5% CO_2-air atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U of penicillin, and 100 μg of streptomycin) (Invitrogen) per ml. For glutathione complementation assays, cells were cultivated in serum-free AIMV (a medium that does not contain glutathione) (Invitrogen) in the presence or in the absence of 500 mM GSH-monoethyl ester (GSH-ME), which was added to the cells before the culture medium in order to ensure an efficient effect.

For indirect protection against apoptosis, supernatants were recovered after 48 h from cultures of infected sheep PBMCs (SI, for supernatant infected) or cells isolated from seronegative animals (SNI, for supernatant noninfected). Conditioned media were cleared by centrifugation and were stored in aliquots at −80°C or used directly to cultivate cells.

Chemicals. To unravel the apoptotic pathways, the following chemicals or proteins were added to the culture medium. (i) Z-IETD-FMK, an irreversible cell permeable inhibitor of caspase 8 that also inhibits granzyme B (Calbiochem), was used at 4 μM after testing a range of concentration of 4 μM, 40 μM, 400 μM, and 4 mM. (ii) Inhibitors of caspases 1 (Z-YVAD-FMK), 2 (Z-VDVAD-FMK), 3 (Z-DEVD-FMK), 5 (Z-WEHD-FMK), 6 (Z-VEID-FMK), 8 (Z-IETD-FMK), 9 (Z-LEHD-FMK), and of all caspases (Z-VAD-FMK) were provided by ImmunoSource (Caspase inhibitory set III) and were used at a concentration of 4 μM. (iii) Concanamycin A (Sigma) selectively inhibits vacuolar H^+-ATPase and eliminates acidification of organelles. The following concentrations were tested: 1 nM, 10 nM, 20 nM, 100 nM, 1 μM, and 10 μM. (iv) Polyclonal activators of viral expression, phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma Aldrich), were used at a concentration of 200 and 565 nM, respectively. (v) APDC (1-pyrrolidinecarbodithioic acid), also called PDTC (Calbiochem), is an inhibitor of NF-κB and interferes with intracellular levels of GSH. (vi) *N*-acetyl-L-cysteine (NAC) (Calbiochem) is an antioxidant that inhibits NF-κB. The following concentrations were tested: 2, 5, and 10 mM. (vii) BAY11-7082 (Calbiochem) selectively and irreversibly inhibits the tumor necrosis factor (TNF)-inducible phosphorylation of IκB-α. This inhibitor was tested at a concentration of 5 μM. (viii) (E)-capsaicin (Calbiochem), a constituent of cayenne pepper, also inhibits NF-κB activation by TNF. The concentrations tested were 10, 100, and 250 μM. (ix) SN50 (Calbiochem) is a cell-permeable inhibitor peptide that interferes with NF-κB nuclear translocation, and it was used at concentrations ranging from 3.6 to 18 μM. (x) N^G-monomethyl-L-arginine (LNMMA; tested at a concentrations of 100 nM and 1, 10, 25, 100, and 250 μM) (Calbiochem) inhibits all three forms of nitric oxide synthase (NOS), whereas NS398 (at 500 nM and 5, 50, and 150 μM) (Calbiochem) selectively impedes cyclooxygenase (COX2). (xi) Ethacrynic acid (Sigma) inhibits glutathione transferase and induces a rapid depletion of GSH. (xii) Recombinant human TNF-α protein (or cachectin) (Calbiochem) induces apoptosis in human PBMCs at doses of 20 to 50 pg/ml and is not species specific (42). (xiii) Anti-TNF-α-neutralizing antibody (fully active at 0.5 ng/ml) was tested at 10 and 20 μg/ml, and the corresponding control rabbit immunoglobulin Gs (IgGs) were purchased from Calbiochem. (xiv) Recombinant protein glutathione *S*-transferase M1-1 (GSTμ) was obtained from Calbiochem and was added to the medium at a dose of 4.5 μg/ml. (xv) DL-buthionine-(*S*,*R*)-sulfoximine (BSO) (Sigma) irreversibly blocks γ-glutamylcysteine synthase and consequently reduces intracellular levels of GSH. BSO was tested at a concentration of 100 μM, 500 μM, 1 mM, and 10 mM. (xvi) GSH-ME (Calbiochem) is a very unstable and cell-permeable derivative of GSH that undergoes hydrolysis by intracellular esterases, thereby increasing reduced glutathione concentration.

Analysis of ex vivo apoptosis. After 18 h of culture, PBMCs were collected, washed twice in PBS–10% FCS, and incubated for 30 min at 4°C in the presence of monoclonal antibody 1H4 recognizing surface IgM (sIgM) as a B-cell marker. PBMCs were then washed again and labeled with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulins (Dako). The labeled cells were fixed with 70% ethanol at −20°C for at least 1 h. After two final washes the samples were treated with RNase A (50 μg/ml) (Sigma Aldrich) for 30 min at 37°C and were incubated for 5 min at room temperature in the presence of 20 μg of propidium iodide (PI) (Sigma Aldrich) per ml. Finally, cells were analyzed by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems). Doublets were excluded from the analysis by using the FL2 area/FL2 width gating method. Ten thousand events (FL1 = 1H4-FITC; FL3 = PI) were collected, and data were analyzed with the CELLQUEST software. Cells in sub-G_1 peak were considered apoptotic.

Measurement of the GSH levels. The GSH levels were determined either directly within the cells or in the supernatants by using the Glutathione Assay kit (Calbiochem). Forty million cells were resuspended in 500 μl of freshly prepared methaphosphoric acid (Sigma) (5% solution in water). After homogenization, the lysate was cleared by centrifugation at 3,000 × *g* for 10 min at 4°C. An aliquot of 200 μl was mixed with buffer no. 3 (200 mM potassium phosphate [pH 7.8] containing 0.2 mM diethylene triamine pentaacetic acid and 0.025% LUBROL) up to a volume of 0.9 ml. Fifty microliters of 12 mM R1 chromogenic reagent (*Calbiochem*) and 50 μl of 30% NaOH were added, mixed thoroughly, and incubated for 10 min at 25°C in the dark. The absorbance was measured at 400 nm.

For measurement in the cell culture supernatants, 200 μl of AIMV-conditioned medium and 40 μl of RPMI-conditioned medium were directly mixed with 700 μl of buffer 3 and the assay was performed as described above.

Measurement of GPx and GR activities. To measure the activities of intracellular glutathione peroxidase (GPx) and glutathione reductase (GR), cells lysates were prepared from freshly isolated PBMCs. Cells were homogenized in cold buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 1 mM dithiothreitol), lysed by a freeze/thaw cycle, and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatants were recovered, and the protein concentrations were determined by the Bradford method. The activities of GPx and GR were quantified by using commercial kits (Cellular Glutathione Peroxidase Assay kit and Glutathione Reductase Assay kit; Calbiochem). For the GPx activity assays, 70 μl of a sample containing 10 μg of protein was diluted in 350 μl of assay buffer (50 mM Tris-HCl [pH 7.6], 5 mM EDTA) and mixed with 350 μl of NADPH reagent (3.2 mM GSH, 0.64 mM NADPH, ≥1.6 U of GR/ml). Finally, 350 μl of 0.704 mM *tert*-butyl hydroperoxide was added and the absorbance were measured at 340 nm every 30 s for 3 min. To measure the activity of GR, 50 μg of protein was diluted in 200 μl of sample diluent (50 mM potassium phosphate buffer [pH 7.5], 2.5 mM EDTA) and mixed to 400 μl of 2.4 mM glutathione disulfide (GSSG). Finally, 400 μl of 0.54 mM NADPH was added and the absorbance at 340 nm was recorded every 60 s for 5 min.

RESULTS

Spontaneous cell death requires a caspase 8-dependent pathway. Different metabolic pathways tightly control apoptosis, a mechanism of active suicide, which is essential during tissue development and which represents a key modulator of

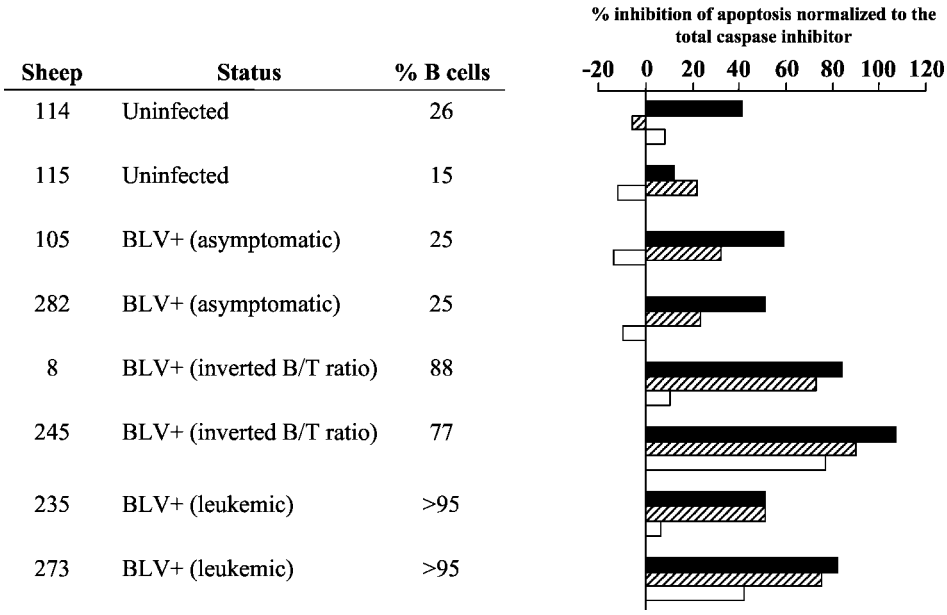


FIG. 1. Caspase inhibitors interfere with apoptosis of PBMCs from sheep. Blood from a series of eight sheep (6 BLV-infected sheep, nos. 8, 105, 235, 245, 273, and 282, and two controls, nos. 114 and 115) was extracted by jugular venipuncture, and PBMCs were isolated by Percoll gradient centrifugation. The percentages of B lymphocytes within the PBMC population were determined by flow cytometry with the anti-IgM 1H4 monoclonal antibody. Uninfected and asymptomatic sheep have less than 26% B cells, whereas in lymphocytic (nos. 8 and 245) and leukemic animals (nos. 235 and 273) this proportion increases up to >95%. Cells were cultivated in RPMI medium supplemented with 10% FCS for 18 h in the presence of peptide inhibitors of the following caspase proteins: 3 (Z-DEVD-FMK; black bars), 8 (Z-IETD-FMK; hatched bars), 9 (Z-LEHD-FMK; white bars), and combined caspases (Total Z-VAD-FMK) at a concentration of 4 μM. The inhibition of apoptosis yielded by Z-VAD-FMK was determined by PI staining of ethanol-fixed cells and was arbitrarily set to 100%. The percentages of decrease in apoptosis in the presence of Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK were normalized to the levels generated by the all-caspases inhibitor Z-VAD-FMK. The data presented are the mean values deduced from three independent experiments, standard deviations being in the 10% range.

cellular homeostasis. During this process, caspases are important cysteine proteases whose activities are critical for the cell death machinery (5, 31, 37, 39). There are two main pathways of caspase activation depending either on death receptor signaling or on cytochrome *c* release from mitochondria. Caspases 8 and 9 appear to be the apical proteases activated in death receptor- and mitochondrial stress-induced apoptosis, respectively. Active caspases 8 and 9 then activate effectors (caspases 3, 6, and 7), leading to onset of the apoptotic process.

To unravel the metabolic pathways involved in spontaneous apoptosis associated with BLV, PBMCs from different sheep were isolated and briefly cultivated in RPMI medium supplemented with FCS and containing a caspase inhibitor (either caspase 3, 8, or 9). The apoptotic levels were determined by PI staining of ethanol-fixed cells, a technique that was previously demonstrated to perfectly fit with the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling procedure (12). Absolute percentages of apoptotic cells within the PBMC population were normalized to the levels yielded by the all-caspases inhibitor. Proportionally, inhibition of the effector caspase 3 efficiently reduced apoptosis between 12% (sheep 115) and 107% (sheep 245) (Fig. 1, black bars). Similarly, impeding the activity of the apical caspase 8 consistently restored cell viability in all but one (sheep 114) culture (hatched bars). In contrast, inhibition of caspase 9 did not protect efficiently against apoptosis, except for cells isolated from sheep 245 and 273 (open bars). The experiment was repeated twice, yielding very reproducible results with individual variations below 10%.

Basically these results suggest that, in total PBMC populations, the main apoptotic pathway occurs via caspase 8 during ex vivo cultivation, although the caspase 9 protease might play an accessory role. However, the effect of caspase 9 inhibitor was not consistently observed in all samples and was therefore not further investigated. To clarify the involvement of caspase 8 in the BLV host cells, apoptosis was analyzed in the B-lymphocyte subset. Dual-flow cytometry of cells labeled with anti-IgM/FITC (FL1) and stained with PI (FL3) revealed that B lymphocytes from infected sheep are relatively less prone to undergoing apoptosis than control animals. Indeed, the ratios of apoptotic B cells versus $G_0/G_1 + G_2/M$ B cells within the PBMC population were 0.95 (40/42) for sheep no. 8 and 3.6 (22/6) for sheep no. 114 (illustrated on the left panels of Fig. 2A). Proportionally, B cells from BLV-infected animals are thus more resistant to cell death, confirming previous observation (12). In the presence of the caspase 8 inhibitor the number of apoptotic B cells was strongly reduced (middle panels of Fig. 2A). This reduction in cell death was, however, less efficient than protection conferred by PMA plus ionomycin (PMA-I), the best antiapoptotic chemicals known to date (right-most panels). To settle the statistical relevance of this phenotype, the effect of the caspase 8 inhibitor was tested on cells isolated from four BLV-infected and four controls in three independent experiments. In general, inhibition of

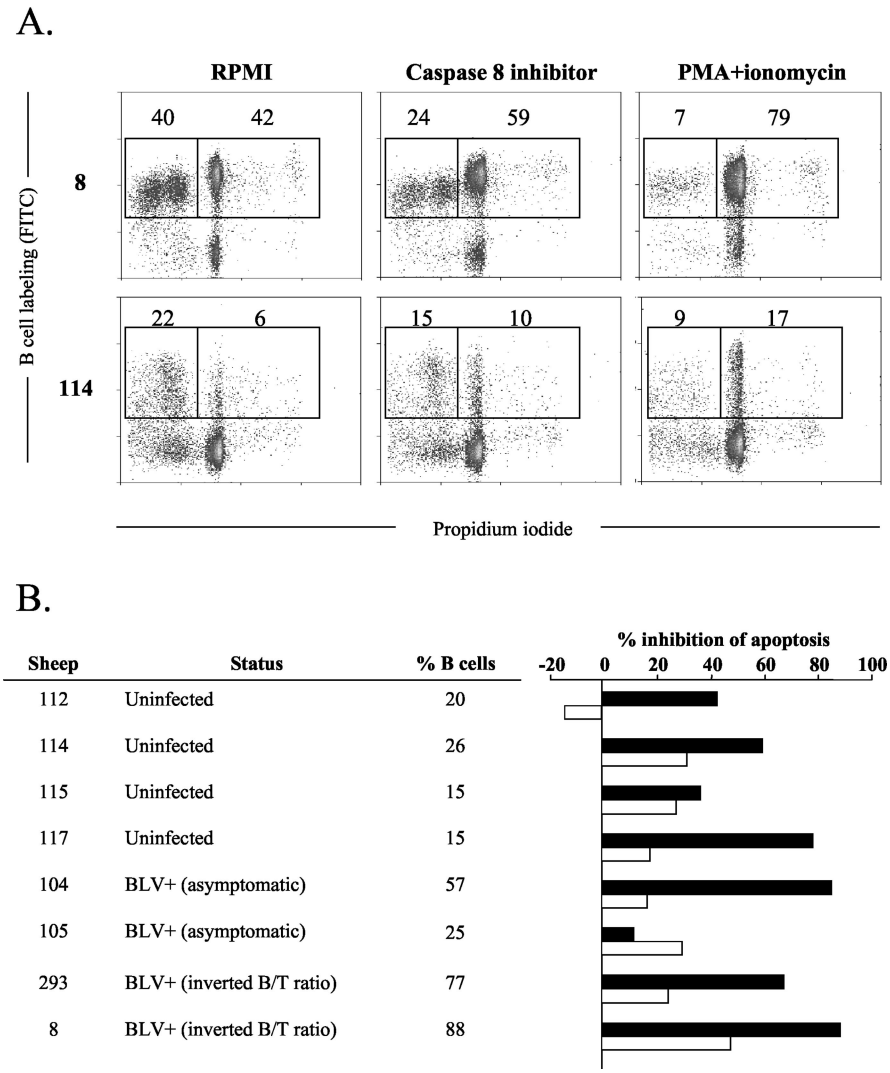


FIG. 2. Caspase 8 inhibitor partly reverts apoptosis of B lymphocytes. PBMCs were isolated ex vivo from BLV-infected (nos. 8, 104, 105, and 293) and control sheep (nos. 112, 114, 115, and 117) and were cultivated alone or in the presence of the caspase 8 inhibitor. As a control, cells were also incubated with two chemicals (PMA-I), allowing maximal protection against apoptosis. After 18 h of culture the percentages of apoptotic B cells were determined by flow cytometry of PI and surface IgM double-stained cells (PI labeling on the *x* axis and B lymphocytes on the *y* axis illustrated in panel A for BLV-infected sheep no. 8 and control animal no. 114). (B) Mean percentages of the inhibition of apoptosis by caspase 8 inhibitor (white bars) or PMA-I (black bars) were deduced from three independent experiments and were normalized to the levels observed in the absence of chemicals (standard deviation being in the 10% range).

caspase 8 reduced apoptosis between 17% (sheep 104) and 48% (sheep 8) (Fig. 2B). Protection against apoptosis was, however, not statistically different in cells isolated from infected or control animals (Student *t* test; $P = 0.46$). This type of cell death inhibition was not observed with other caspase inhibitors (i.e., caspases 1 [Z-YVAD-FMK], 2 [Z-VDVAD-FMK], 5 [Z-WEHD-FMK], and 6 [Z-VEID-FMK]) and was not induced by concanamycin, which interferes with the perforin pathway and cytotoxic T-lymphocyte (CTL)-dependent killing (data not shown).

We thus conclude that caspase 8 is involved in spontaneous apoptosis of cultivated ovine B lymphocytes regardless of viral infection.

APDC and ethacrynic acid inhibit indirect protection against apoptosis. With the aim of further understanding the apoptotic process, a series of inhibitors was randomly screened for their ability to interfere with indirect protection against cell death, a mechanism illustrated in Fig. 3A. When cells were cultivated in a supernatant from infected sheep PBMCs, the number of apoptotic B lymphocytes was drastically reduced compared to that in unconditioned RPMI medium (6% versus 32%). Under the same experimental conditions, this inhibition of apoptosis was not induced (at least to the same extent) by conditioned medium from PBMCs isolated from noninfected sheep (Fig. 3A, right-most panel). As a preliminary experiment, the first obvious assay was to test the role of viral proteins in this inhibition phenotype. Supernatants of fetal lamb kidney (FLK) cells containing similar titers of BLV virions (FLK-BLV cell line) did not reduce apoptosis under similar conditions, indicating that the protective factor was not viral protein (data not shown).

Among a series of inhibitors interfering with metabolic path-

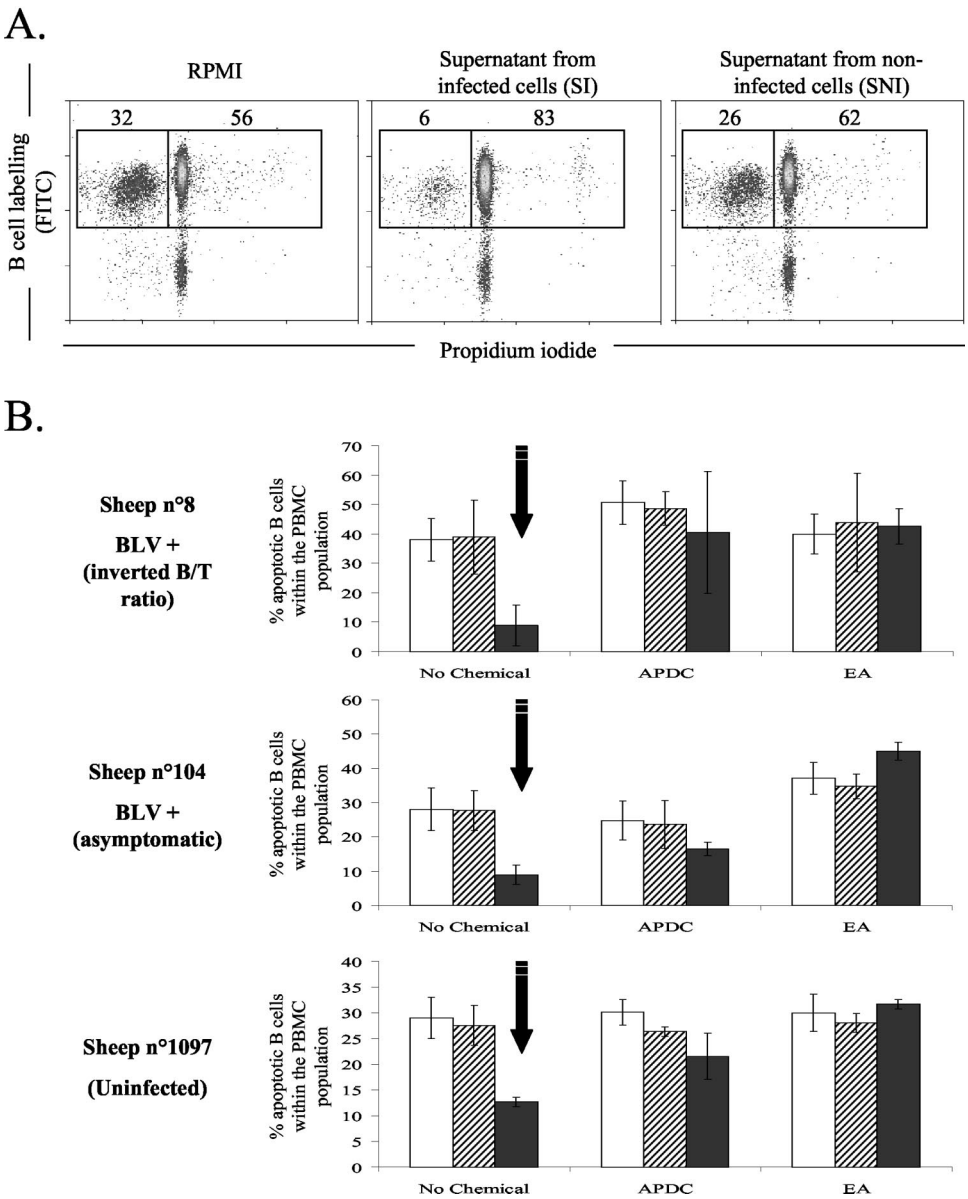
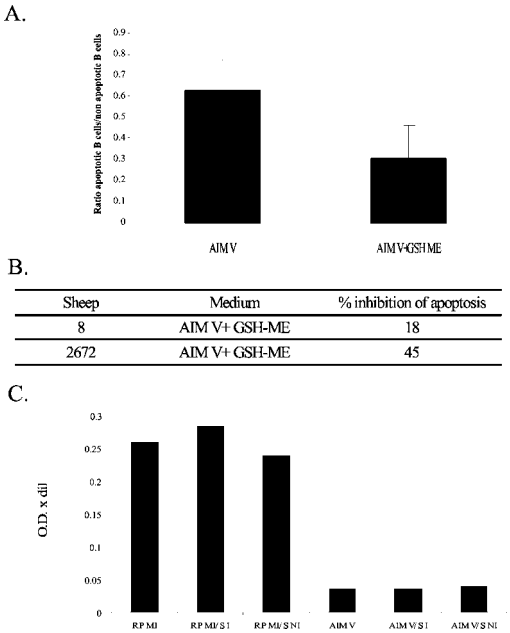


FIG. 3. APDC and ethacrynic acid inhibit indirect protection against apoptosis. (A) PBMCs from sheep 8 were cultivated for 18 h in RPMI medium (left-most panel) or in medium conditioned with cells isolated from a BLV-infected sheep (SI, middle panel) or a control animal (SNI, right panel). The percentages of apoptotic B cells within the PBMC population based on B-lymphocyte/propidium iodide staining were determined as described in the legend to Fig. 2. (B) Recapitulative graphs summarizing the percentages of apoptotic B cells within the PBMC population in different culture media (RPMI, white bars; SNI, hatched bars; SI, black bars) without chemical or containing APDC at a concentration of 10 μM or ethacrynic acid (EA) at 5 μM. The data are the mean values and their respective standard deviations deduced from three independent experiments and performed with cells isolated from two BLV-infected sheep (no. 8 and 104) and one control (no. 1097).

ways (see Materials and Methods and Fig. 7), we identified APDC, also called PDTC, as a chemical specifically abrogating the antiapoptotic effect (Fig. 3B). Indeed, in the presence of an optimal dose of 10 μM APDC, the percentage of apoptotic B cells in control (RPMI and SNI) or BLV-infected (SI) supernatants were similar. The protective effect induced by SI medium in the absence of chemical was thus completely reverted. These observations were made with cells isolated from three sheep at different stages of pathogenesis: one prelymphocytic sheep (no. 8), one asymptomatic (no. 104) and one control (no. 1097) (Fig. 3B). Because APDC was initially characterized as an inhibitor of NF-κB, we tested two other chemicals interfering with this transcription factor (i.e., NAC and SN50), which unexpectedly did not abrogate the protective effect (data not shown). Furthermore, a series of other compounds interfering with the TNF/NF-κB/NOS/COX2 pathway [i.e., BAY11-7082, (E)-capsaicin, LNMMA, and NS398; see Fig. 7] did not selectively impede protection against apoptosis, although two of them (BAY11-7082 and NS398) were either inactive or directly toxic to the cells (data not shown; see Materials and Methods for experimental conditions).

Together these results indicate that APDC does not modu-



Sheep	Medium	% inhibition of apoptosis
8	AIM V+ GSH-ME	18
2672	AIM V+ GSH-ME	45

FIG. 4. Exogenous cell-permeable GSH protects from apoptosis. (A) PBMCs were isolated from a BLV-infected sheep (no. 1095) and were cultivated for 18 h in glutathione-free AIMV medium with (AIMV+GSH-ME) or without (AIMV) supplement plus cell-permeable GSH-ME at a concentration of 500 μM. The data representing the ratio of apoptotic cells within the B-lymphocyte population are the results of three independent experiments. (B) Percentages of inhibition of apoptosis affecting B lymphocytes in PBMCs isolated from BLV-infected sheep no. 8 and 2672. Data (in percent inhibition of apoptosis) were deduced by normalization of the apoptotic levels obtained in the presence of GSH-ME in the medium with those detected in its absence. (C) Measurement of reduced glutathione in protective (RPMI-SI or AIMV-SI medium conditioned with cells isolated from BLV-infected sheep) or control supernatants (RPMI, RMPI-SNI, or AIMV, AIMV-SNI). The data (in optical densities [O.D.]) were standardized for the dilution factor (dil).

late apoptosis via NF-κB, at least under spontaneous ex vivo cell culture conditions. APDC has, in fact, been described both as an inducer or an inhibitor of apoptosis by modifying the levels of intracellular reduced glutathione (GSH) (30, 51). Indeed, another compound inducing a rapid depletion of GSH, ethacrynic acid (36), also reverted the protection phenotype (Fig. 3B).

We conclude that two chemicals, APDC and ethacrynic acid, specifically inhibit indirect protection against apoptosis, possibly via modulation of intracellular glutathione.

Decrease in apoptosis parallels decrease in glutathione intracellular levels. Reduced glutathione is a tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways as well as in the antioxidant systems of most aerobic cells (13, 43). To test the ability of GSH to inhibit apoptosis, PBMCs were isolated from a BLV-infected sheep (no. 1095) and cultivated in glutathione-free AIMV medium. Addition of cell-permeable GSH-ME significantly reduced cell death ($P < 0.05$) (Fig. 4A). It thus appears that primary B lymphocytes can be protected by exogenously provided reduced glutathione, as expected. Similar results were obtained with cells from other sheep (nos. 8 and 2672), indicating that this protection was independent of the stage of infection (presented as percentages of inhibition of apoptosis in Fig. 4B). However, the levels of GSH were not significantly different in protective and in control supernatants (compare RPMI-SI or AIMV-SI with RPMI, RMPI-SNI, or AIMV, AIMV-SNI, respectively), suggesting that extracellular reduced glutathione was not by itself an inhibitor of apoptosis (Fig. 4C).

To correlate the intracellular GSH levels with apoptosis, PBMCs were isolated from BLV-infected sheep (no. 8) and cultivated in preconditioned media as described above. In three independent experiments the levels of reduced glutathione were consistently enhanced (optical density of 0.078) within cells cultivated in SI supernatant compared to those cultivated in the control media (0.039 in SNI and 0.046 in RPMI) (Fig. 5). The increase in GSH content was inversely correlated to the number of apoptotic cells (10.9, 40.3, and 48.2% in SI, SNI, and RPMI, respectively; Fig. 5B). In other words, protection against cell death conferred by the infected supernatant (SI) parallels the intracellular glutathione levels. Most importantly, incubation of cells with PMA-I very efficiently inhibited apoptosis but did not induce GSH, indicating

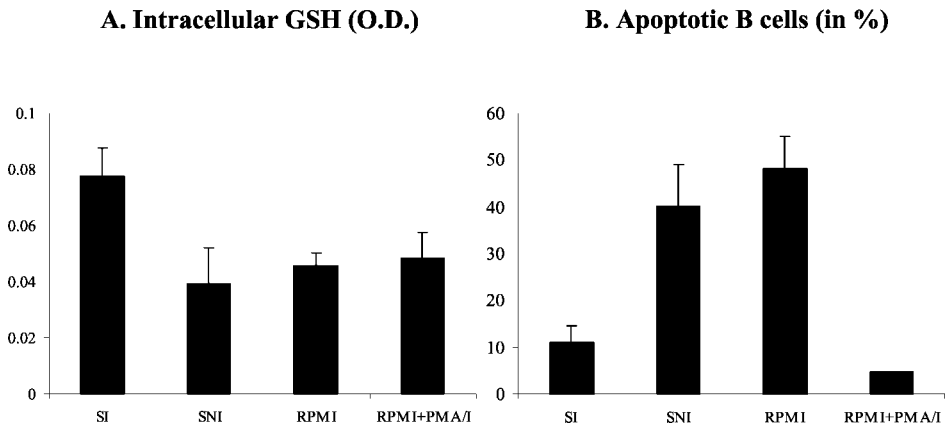


FIG. 5. GSH is a key factor for indirect protection against apoptosis. Cells from sheep no. 8 were cultivated for 18 h in different media (SI supernatant conditioned with cells isolated from BLV infected sheep, control SNI medium, and RPMI with our without PMA-I). The intracellular GSH levels (A) were determined from 4×10^7 cells, and the percentages of apoptotic B lymphocytes (B) were measured in parallel by using a proportion of them (4×10^6 cells). O.D., optical density.

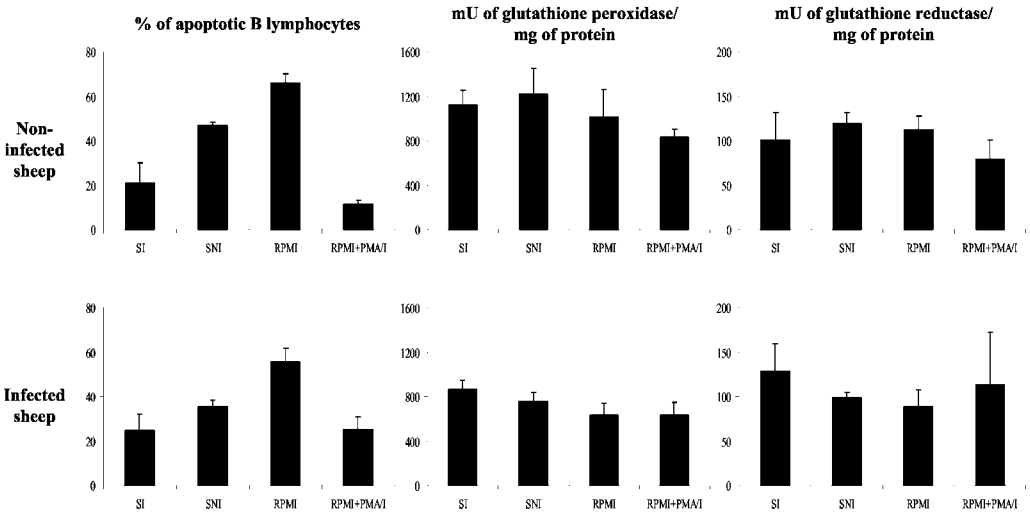


FIG. 6. Protection against apoptosis does not correlate with a difference of GSH peroxidase and GSH reductase activities. PBMCs from one BLV-infected sheep (no. 2675) and one noninfected sheep (no. 2274) were isolated and cultivated for 18 h in different culture media (RPMI, SNI, SI, and RPMI plus PMA-I). Apoptotic B lymphocytes were detected by flow cytometry after PI-surface IgM labeling. The cell lysates containing 0.01 and 0.05 mg of protein were used to measure the activities of GSH peroxidase and GSH reductase, respectively. The results shown in the graphs are means of three independents experiments.

that the effect was specific to the SI-conditioned medium (Fig. 5). Similar results were repeatedly obtained with cells isolated from another sheep at the leukemic stage (GSH levels of 0.078, 0.065, and 0.028, corresponding to apoptotic percentages of 16, 29, and 16% in SI, RPMI, and RPMI plus PMA-I, respectively). Again, the highest GSH levels were thus found in the SI medium but not in RPMI plus PMA-I medium, although the apoptotic rates were similar. Finally, inhibition of apoptosis induced by conditioned medium from lymphocytotic cattle lymphocytes did not correlate with GSH levels in cultures of bovine PBMCs, indicating that the effect was species specific (data not shown).

Apoptotic levels do not correlate with activities of GSH peroxidase and GSH reductase. Glutathione acts as a substrate for a variety of enzymes, mainly GPx, which catalyzes the reduction of hydrogen peroxide into water and oxidation of GSH into GSSG (oxidized glutathione). These reactions lead to the detoxification of peroxides and protect cells from free radical damage. Inversely, GR catalyzes the reduction of GSSG to GSH, ensuring the glutathione redox cycle and maintaining adequate levels of GSH.

To unravel the mechanisms mediating the levels of intracellular glutathione, the enzymatic activities of GPx and GR were measured in cells cultivated under different conditions. For this purpose the PBMCs from an infected (no. 2675) and a control (no. 2274) sheep were isolated and cultivated for 18 h in four different media (SI, SNI, RPMI, and RPMI with PMA-I). The activities of GPx and GR as well as the levels of apoptosis were then determined from the PBMC cultures (Fig. 6). No difference was observed between GPx and GR activities in cells cultivated in infected supernatant (SI) and those in other media (SNI, RPMI, and RPMI with PMA-I), indicating that the higher levels of intracellular GSH associated with protection against apoptosis were not due to different activities of these enzymes.

DISCUSSION

The aim of this report is to cast light onto the apoptotic process associated with BLV as a comparative model for the related HTLV-1. Our experimental system, which is based on the use of freshly isolated primary cells from infected sheep, is thought to closely mimic the mechanisms occurring in vivo. A first contribution concerns the proteases that are required for completion of spontaneous ex vivo cell death. We show that inhibition of caspases 8 and 3 partially restricts cell death and reveals an apoptotic pathway depending on receptor signaling (Fig. 1 and 2; also see Fig. 7). Although the implication of caspase 9 could not be completely excluded in total PBMC populations (for instance, sheep nos. 245 and 273; Fig. 1), it clearly appears that signaling via cytochrome *c* release from mitochondria is not a major initiator step during spontaneous apoptosis in B lymphocytes. It is still possible that caspase 9 is involved during the full onset of apoptosis, but inhibition of its activity is not sufficient to block the initiation of the process. In this context, caspase 8 is known to cleave Bid, the proapoptotic Bcl2 member, and trigger cytochrome *c* release, providing a shortcut to mitochondria-dependent apoptosis (31). However, under our experimental conditions we could exclude the perforin-dependent cytotoxic pathway by using the granzyme B inhibitor, concanamycin A. Our results thus clearly reveal that caspase 8 is the predominant initiator protease implicated in the apoptotic process of ex vivo-isolated ovine B lymphocytes. A first direct conclusion of the involvement of this particular protease is that the death process is apparently not associated with the B-cell receptor (BCR) signaling known to be independent of caspase 8 activity (4). Instead, caspase 8 can be activated by three types of membrane-associated receptors: TNF-R1, Fas/CD95, and TRAIL-R2/DR5, all belonging to a structurally related and still growing family of proteins (5). It is noteworthy that the TNF pathway has an essential role in

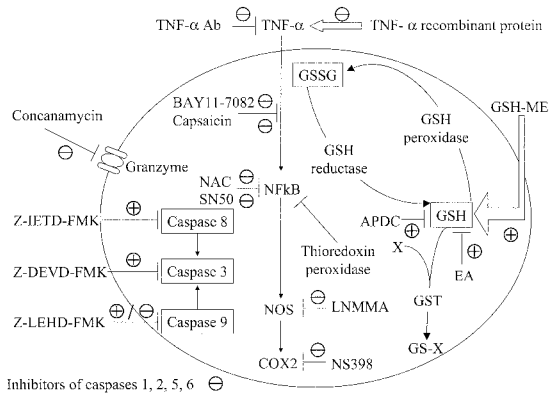


FIG. 7. Recapitulative scheme of the pathways analyzed in this study. A series of inhibitors (—|) and two activators (arrows for GSH-ME and TNF-α recombinant protein) are represented in regard to the cellular enzyme that is specifically targeted. A plus sign means that the inhibitor or the activator affects the apoptotic ratios of ovine B lymphocytes from both infected and control sheep. Conversely, a minus sign means that the inhibitor or activator does not specifically alter cell death over a broad range of concentrations. A plus/minus means that the inhibitor is able to inhibit apoptosis in some samples. The apoptotic process of ovine B lymphocytes involves a caspase 8-dependent pathway (inhibited by Z-IETD-FMK) and the caspase 3 effector (Z-DEVD-FMK). The caspase 9 inhibitor (Z-LEHD-FMK) was effective in a minority of cases, and concanamycin did not interfere with apoptosis, indicating the absence of a cytotoxic response under the experimental conditions used. The following series of compounds interfering with the TNF-α/NF-κB/NOS/COX2 pathway was used: TNF-α Ab (neutralizing antibody or recombinant protein), BAY11-7082, capsaicin, NAC, SN50 (NFκB inhibitory peptide), LNMMA (inhibiting NOS), and NS398 (an inhibitor of COX2). None of these compounds selectively impedes indirect protection against apoptosis conferred by a medium conditioned by BLV-infected cells. Reduced glutathione plays a key role in this process because (i) membrane-permeable GSH-ME restricts apoptosis and (ii) APDC and ethacrynic acid (EA) known to decrease GSH levels eliminate indirect protection against apoptosis. The activities of two enzymes implicated in GSH regulation (GSH reductase and GSH peroxidase) are unaltered during this process. Two other enzymes linked to the NF-κB and GSH pathways, i.e., thioredoxin peroxidase and GST, respectively, are indicated. GST catalyzes the conjugation of the thiol group of reduced glutathione to xenobiotics (X), yielding GS-X.

apoptosis as well as in cell survival and that the outcome of its signaling is modulated by many environmental and cell-type-specific factors (3, 50). Based on two inhibitors (BAY11-7082 and capsaicin), our data provide evidence that the TNF-α cascade is not involved in spontaneous apoptosis associated with BLV (Fig. 7). Furthermore, neither the depletion of conditioned media by means of an anti-TNF antiserum nor the complementation of TNF-α recombinant protein had any effect on cell death (data not shown). Although the role of CD95 and TRAIL-R2 could be determined, for example, by using soluble receptor Fc proteins as decoys for their natural ligands (4), we did not further investigate this pathway because the apoptotic process does not appear to be modulated by viral infection (Fig. 2B). Indeed, both cells from virus-infected animals and normal lymphocytes are equally susceptible to the Z-IETD-FMK inhibitor, indicating that caspase 8-dependent initiation of apoptosis was not linked to the presence of BLV. Additional theoretical evidence is also provided by the lack of homology between any BLV open reading frame and anti-apoptotic proteins, such as v-FLIPs (viral FLICE/caspase 8 inhibitory proteins). It thus appears that BLV does not inhibit apoptosis by modulating death receptor signaling.

Based on several criteria (absence of specific response to BAY11-7082 or capsaicin; lack of interference of an anti-TNF antiserum; no phenotype associated with recombinant protein added to the cultures; Fig. 7), our data show that the TNF-α pathway is not involved in ex vivo spontaneous cell death. It has been well characterized that NF-κB exerts an essential function in B lymphocyte regulation downstream of this receptor (20), and we were in fact quite surprised that NF-κB did not play a role in apoptosis. Indeed, NAC efficiently inhibiting NF-κB as revealed by gel shifts (data not shown), and SN50, a cell-permeable peptide that interferes with its nuclear translocation, did not prevent cell death. Further downstream of this pathway, both LNMMA and NS398, inhibiting NOS and COX2, respectively, were also devoid of specific proapoptotic activity (Fig. 7), although these enzymes have been implicated in cell death in other systems, including HTLV-1 (28, 44, 47).

Together, our results thus reveal that the metabolic pathways initiated during apoptosis of cells isolated from BLV-infected animals closely resemble the normal processes occurring in the absence of virus. We also show that the quantitative difference in spontaneous cell death conferred by a medium conditioned by BLV-infected cells relies on another important modulator of apoptosis: reduced glutathione. This small molecule is the most abundant thiol-containing compound of the cell and is involved in a large variety of metabolic pathways as well as in the antioxidant system. Exposure to reactive oxygen species such as hydrogen peroxide (H_2O_2) or nitric oxide (NO) initiates apoptosis in different cell types and, conversely, many apoptotic inducers, like growth factor deprivation, UV irradiation, and cytotoxic drugs, cause oxidation (8, 46). Similarly, we hypothesize that the observed rise in intracellular GSH specifically associated with BLV is also linked to the decrease of oxidation and subsequent impairment of apoptosis. In fact, reduced GSH is converted to its oxidized form (GSSG) by cytosolic selenoenzymes, the GPx's. Inversely, GSSG is reduced to generate GSH by GR in the presence of NADPH. Intracellular reduced glutathione levels can thus be modulated by the activities of both peroxidase and reductase enzymes. However, our results demonstrated that these enzymes are apparently not implicated in the indirect protection against apoptosis conferred by the infected supernatant (Fig. 6). Therefore, our model (Fig. 7) postulates that GST plays a key role in modulation of GSH as also supported by pharmacological inhibition by ethacrynic acid and APDC.

It is noteworthy that protection conferred by glutathione is highly dependent on the experimental conditions used upon initiation of the short-term culture. Indeed, the cell-permeable form of GSH (GSH-ME) must be added to the cells prior to addition to the culture medium in order to yield efficient protection, and this requirement similarly holds true for the anti-caspase 8 inhibitor (Z-IETD-FMK). Inversely, slow depletion of GSH production by means of BSO, a specific inhibitor of γ-glutamyl-cysteine synthetase, or addition of NAC, another antioxidant and precursor of glutathione, does not decrease apoptosis (data not shown). Together these observations suggest that the decision to commit cell suicide is taken very early after being put in contact with culture medium, and, GSH

depletion accordingly is known as an early event in apoptosis (25). Furthermore, the reducing activity of another antioxidant, NAC, is not sufficient to inhibit cell death, providing further support for a specific role of glutathione. Additional evidence for specificity is also supported by the lack of response to polyclonal activators (PMA I) that inhibit apoptosis without raising GSH levels, in contrast to BLV-conditioned media (Fig. 5). However, because BLV-containing supernatants from FLK cells do not protect from apoptosis, it appears that the protective factor is not the virus itself. Examples of direct regulation are provided, for instance, by the GPx of molluscum contagiosum virus, which protects from peroxide apoptosis, and CrmA, a cow pox virus cytokine modifier protein inhibiting depletion of GSH induced by TNF-α (1). In contrast, the process of GSH modulation in the BLV system appears to be rather indirect by a still-undefined virus-associated mechanism. Importantly, the levels of GSH in the supernatants obtained from infected and control cells are similar, indicating that the protective factor is not extracellular GSH itself. Direct protection by glutathione or other reductors has been frequently described for several malignancies, including leukemia, essentially revealing the role of the antioxidant process during apoptosis (5, 54, 56). In contrast, a specific increase of GSH in cells from lymphocytic patients has been far less documented (23, 32), and we provide here an example of a mechanism linking intracellular GSH to apoptosis associated with BLV infection.

To summarize, our results show (i) that addition of synthetic reduced glutathione inhibits cell death, (ii) that medium conditioned with primary lymphocytes from BLV-infected sheep concomitantly induces intracellular GSH levels and abrogates spontaneous apoptosis, (iii) that inhibitors shown previously (APDC and ethacrynic acid) to lower intracellular concentration of GSH reverse protection of apoptosis conferred by a conditioned medium from infected sheep PBMCs, and (iv) that inhibition of apoptosis is not linked to a difference of the GPx or GR activities, suggesting that other pathways, such as detoxicative depletion by glutathione transferase, modulate the levels of GSH. Together these data demonstrate that GSH is a key factor involved in indirect protection against apoptosis associated with BLV-infected cells.

The role of GSH in BLV-associated apoptosis could have consequences for pathogenesis induced by the related HTLV-1 and treatment of ATL. Indeed, besides a mechanism of direct CTL killing (18, 19), regulation of the antioxidant equilibrium appears to be an important parameter of the apoptotic process, with HTLV-1 immortalized cell lines requiring thiol compounds for efficient growth in vitro (24, 26, 40, 45). Although several therapeutic protocols do exist and yield some encouraging results, ATL carries a very poor prognosis due to an intrinsic resistance of leukemic cells to conventional or even high doses of chemotherapy (2). One of the last advances in ATL treatment is based on the combination of arsenic trioxide (As_2O_3) and IFN-inducing cell cycle arrest and apoptosis of HTLV-1-positive cells. Because intracellular glutathione content has a decisive effect on As_2O_3-induced apoptosis (10, 16), specific targeting of GSH could help in increasing the efficiency of this type of treatment.

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