Journal of General Virology (1997), **78**, 153–162. Printed in Great Britain

Bovine leukaemia virus-induced lymphocytosis in sheep is associated with reduction of spontaneous B cell apoptosis

Isabelle Schwartz-Cornil,[1] Nathalie Chevallier,[1] Catherine Belloc,[1] Danielle Le Rhun,[1] Véronique Lainé,[1] Madeleine Berthelemy,[1] Ana Mateo[2] and Daniel Levy[1]

[1] URA INRA d'Immuno Pathologie Cellulaire et Moléculaire, 7 avenue du général de Gaulle, 94704 Maisons-Alfort cedex, France
[2] Universitad de Cordoba, Facultad de Veterinaria, Avenida de Medina Azahara, 9, 14005 Cordoba, Spain

Experimental inoculation of sheep with bovine leukaemia virus (BLV), a retrovirus homologous to the human T-lymphotropic virus type 1 (HTLV-1), induces a chronic expansion of the B lymphocyte population (persistent lymphocytosis) and the development of a B cell leukaemia/lymphosarcoma syndrome. To gain insight into the mechanisms of BLV-induced lymphocytosis, we tested B cell survival capacity and cycling activity in peripheral blood mononuclear cells (PBMCs) from lymphocytotic, asymptomatic and control sheep. Interestingly, B cells from lymphocytotic sheep presented a lower level of spontaneous apoptosis (29%) in *ex vivo* cultures compared to that obtained with infected asymptomatic (42%) and control (57%) sheep PBMCs. Virus capsid (CA) synthesis was mainly found among surviving B cells and the percentage of CA-producing B cells correlated with the extent of B cell survival, indicating that BLV replication in B lymphocytes may promote protection from cell death. B cell survival was not linked with increases in expression of Bcl-2 mRNA or membrane leukosialin (CD43), although both are documented to be involved in some aspects of the B cell life-span. Finally, cell cycle analyses in freshly isolated PBMCs from lymphocytotic sheep revealed a slightly increased proportion of B cells in S phase compared to controls. Altogether, these data suggest that both BLV-induced B cell proliferation and extended survival are involved in the lymphocytotic stage encountered in BLV infection in sheep.

Introduction

Bovine leukaemia virus (BLV) is a complex lymphocytotropic retrovirus that is structurally and genetically similar to human T-lymphotropic virus types 1 and 2 (HTLV-1 and -2). HTLV-1 infection is associated with T cell leukaemia whereas BLV infection in cattle induces alterations in B lymphocyte homeostatic regulation (Schwartz & Levy, 1994). Several months to years after infection, 30 to 70% of the cows present persistent lymphocytosis, a condition that indicates a progressive expansion of mature $CD5^+$, $CD11b^+/c^+$ B lymphocytes (Matheise *et al.*, 1992); 1 to 6 years later, around 1% of the infected cattle develop a B cell lymphosarcoma (Schwartz & Levy, 1994). Experimental inoculation of BLV into sheep induces a B cell lymphosarcoma sometimes associated with a B cell leukaemia at a higher incidence and after a shorter latency period than in cattle (Mammerickx *et al.*, 1988); in addition, BLV-infected sheep also frequently show a non-neoplastic expansion of the B lymphocyte population (Schwartz *et al.*, 1994; Birkebak *et al.*, 1994; Murakami *et al.*, 1994).

In both sheep and cattle, BLV is mainly found integrated in the B cell population but expression of the virus genome is repressed *in vivo* and sensitive methods such as RT–PCR are needed to detect the virus RNAs (Alexandersen *et al.*, 1993). Strikingly, virus latency is alleviated when the PBMCs are cultured *ex vivo* and virus gene expression culminates 24 h after culture initiation (Powers & Radke, 1992). HTLV similarly displays a repressed/derepressed status *in vivo* and *ex vivo*, respectively (Persaud *et al.*, 1995).

In both BLV and HTLV, two virus proteins participate in the regulation of virus gene expression: the Tax proteins (BLV, p38; HTLV-1, p40) and the Rex proteins (BLV, p18; HTLV-1, p27). The Tax proteins transactivate virus gene expression through three 21 bp repeats centred on a cAMP-responsive element located in the virus long terminal repeats (Katoh *et al.*, 1989; Jeang *et al.*, 1988). The Rex proteins control virus mRNA processing and modulate the accumulation of full-

Author for correspondence: Isabelle Schwartz-Cornil.
Fax +33 1 43 96 71 25. e-mail schwartz@jouy.inra.fr

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length and singly spliced mRNA (Derse, 1988; Felber *et al.*, 1989); the Rex proteins thus appear to be crucial to the expression of virus structural proteins.

These regulatory proteins may participate in virus pathogenesis. In this respect, the BLV Tax protein has been shown to immortalize rat embryo fibroblasts (Willems *et al.*, 1990) and HTLV Tax transforms cord blood T lymphocytes (Grassmann *et al.*, 1989), indicating that the Tax proteins have oncogenic properties. However, this point of view is complicated by conflicting data reporting that HTLV Tax either promotes (Chlichlia *et al.*, 1995; Yamada *et al.*, 1994; Fujita & Shiku, 1995) or prevents apoptosis (Copeland *et al.*, 1994) in *in vitro* experimental systems. In the case of BLV, we recently showed that whole BLV and Tax cDNA expression prevent density-induced apoptosis in B lymphoid cell lines (I. Schwartz-Cornil, D. Le Rhun, I. Oswald, V. Lainé, L. Willems, B. Schwartz and D. Levy, unpublished results). However, these data emerge from *in vitro* experiments involving cell lines and apply to specific conditions that may not be relevant in the *in vivo* situation. In order to shed light onto the mechanisms of BLV-induced B lymphocytosis, we assessed the effects of BLV infection on B cell survival and proliferation by measuring *ex vivo* spontaneous apoptosis and the cell cycling activity in freshly isolated B lymphocytes from uninfected and BLV-infected sheep.

Methods

■ **Experimental inoculation of BLV into sheep.** The sheep used in this study were prealpine castrated males housed at the Ecole Nationale Vétérinaire in Maisons-Alfort, France. Three 4-year-old sheep were used as controls. The BLV-infected sheep were inoculated intraperitoneally at 6 months of age with 5×10^7 PBMCs from a lymphocytotic sheep that had been infected with PBMCs from a naturally infected lymphocytotic cow and that eventually died with disseminated lymphosarcoma. Four sheep (105, 120, 142 and 121) had been infected for 4 years and five sheep (35, 99, 79, 85 and 92) for 2 years.

■ ***Ex vivo* cultures of PBMCs and apoptosis detection.** PBMCs were collected using Percoll gradient centrifugation (Cornil *et al.*, 1988). Cells were seeded at a density of 5×10^6 PBMCs per ml in RPMI-1640 plus 10% foetal calf serum and incubated for 24 h in 5% CO_2 at 37 °C. Apoptosis was estimated using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique, performed according to the manufacturer's recommendations (In Situ Cell Death Detection Kit; Boehringer Mannheim). The TUNEL reaction allows the labelling of DNA strand breaks by incorporation of fluorescein isothiocyanate (FITC)-labelled dUTP on the 3′-OH DNA residues with terminal transferase. The fluorescent dead cells were subsequently analysed using a FACScan machine equipped with a doublet discrimination module (Lysis II software; Becton Dickinson).

Alternatively, apoptosis was measured with propidium iodide staining of the B cell nuclei that appeared subdiploid in FACScan analysis. Cells were fixed in 70% ethanol, washed in PBS, treated with 50 µg/ml RNase for 30 min, incubated for 10 min in 20 µg/ml propidium iodide and analysed using a FACScan with exclusion of cell doublets.

■ **Monoclonal antibodies (MAbs) and flow cytometry analyses.** The IgM DU2-104 that recognizes sheep CD21 was provided by W. Hein (Basel Institute for Immunology, Basel, Switzerland). In double staining control experiments, we verified that DU2-104 labelled all the immunoglobulin-bearing cells. For labelling B cells, 5×10^6 PBMCs were incubated with 50 µl DU2-104 hybridoma culture supernatant for 30 min, then washed in PBS containing 2% horse serum and further incubated with a 1:50 dilution of phycoerythrin (PE)- or FITC-conjugated $F(ab')_2$ goat anti-mouse IgM antibody (Jackson ImmunoResearch Laboratories). Cells were then washed in PBS and analysed using flow cytometry or processed for subsequent detection assays (TUNEL or propidium iodide staining).

Two pools of anti-CA and anti-envelope gp51 (Env) IgG1 MAbs were kindly provided by D. Portetelle (Gembloux, Belgium). PBMCs (5×10^6) were fixed in 2% freshly made paraformaldehyde and permeabilized in 0·1% Triton X-100 in acetate buffer. Permeabilized cells were incubated for 30 min with a 1:100 dilution of anti-CA or anti-Env antibodies in PBS plus 1% BSA. After washing, cells were incubated for 30 min in a 1:50 dilution of PE-labelled goat anti-mouse IgG1 antibody (Jackson ImmunoResearch Laboratories). Cells were finally washed in PBS and processed for TUNEL.

The sheep CD43 determinant was detected with the IgG1 Co-44B8 MAb (Oscar Pintado *et al.*, 1995) and the sheep CD5 molecule was revealed with the IgG2a ST1-A MAb (Keech & Brandon, 1991). Dual labelling with IgM DU2-104 and IgG1 Co-44B8 or IgG2a ST1-A was performed by incubating the PBMCs with 50 µl of each hybridoma supernatant; after washing, cells were incubated with a 1:50 dilution of PE-coupled goat anti-mouse IgM and FITC-coupled goat anti-mouse IgG1 or anti-mouse IgG2a (Caltag Laboratories). Cells were washed and analysed using flow cytometry.

■ **Cell cycle analyses.** Freshly collected PBMCs were labelled for B cell detection with DU2-104 followed by an FITC-conjugated goat anti-mouse IgM, washed in PBS and fixed in 70% cold ethanol for 10 min, washed twice in PBS, incubated in 50 µg/ml RNase A for 30 min at 37 °C, washed in PBS and incubated in 20 µg/ml propidium iodide for 10 min before FACS analysis. The cell cycle was analysed using the Lysis II software on gated B cells with exclusion of cell doublets.

■ **Bcl-2 mRNA detection.** B cells were purified using DU2-104-coated immunomagnetic beads as previously described (Schwartz *et al.*, 1994) and the isolated B cell population was over 97% pure. The B cell-coated beads corresponding to 1×10^5 lymphocytes were treated with a 4 M guanidium thiocyanate solution. The lysates were incubated for 5 min with 10 µl glass milk from the RNaid RNA Extraction Kit (BIO 101). The RNA-coated glass milk was washed five times with the RNaid washing buffer and the RNA was eluted at 60 °C in DEPC-treated water. An aliquot of RNA corresponding to 4×10^4 cells was DNase-treated and divided into two portions; one was reverse-transcribed using random hexamers and 12·5 units avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and the other was directly used in the PCR reactions to check for DNA contamination in the samples. In order to perform standard semi-quantitative PCR (Rashtchian, 1994), the resulting cDNA was divided into two portions (10^4 cell equivalent), one for the housekeeping gene *G3PDH* amplification, the other for *bcl-2* amplification. The detection of *G3PDH* cDNA is used to normalize *bcl-2* detection between samples. *G3PDH* oligonucleotides that cross react between species were utilized (forward: 5′ GACCCCTTCATTGACCTCAACTACA 3′; reverse: 5′ CATGTGGGCCATGAGGTCCACCAC 3′). Twenty-seven PCR cycles were performed as follows: 1 min at 91 °C, 1 min at 60 °C and 1 min at 71 °C. The *G3PDH* PCR products (880 nt) were transferred onto a nylon membrane and revealed with a *G3PDH* digoxigenin-labelled cDNA probe as recommended by the manufacturer (Boehringer Mannheim). The PCR conditions were strictly defined such that a linear relationship between initial cell number and

G3PDH signal intensity was obtained. The *bcl-2* detection was similarly performed on the other half of the cDNA. For *bcl-2* PCR, we used oligonucleotides designed from mouse and human *bcl-2* sequences (forward: 5′ TGTCACAGAGGGGCTACGAGTGGG 3′; reverse: 5′ GGTTCAGGTACTCAGGTCATCCACA 3′). The PCR cycles were 91 °C for 1 min, 50 °C for 1 min and 71 °C for 1 min and were repeated 35 times. The 477 bp PCR products were blotted onto a nylon membrane and were revealed using a digoxigenin-labelled mouse *bcl-2* cDNA-derived probe. This probe was obtained by PCR from mouse genomic DNA using the oligonucleotides as described above. The blot washing was performed at 65 °C in 1 × SSC and 0·1% SDS for 20 min. A linear correlation between the initial input of cells and the PCR product signal was obtained in these experimental conditions. The densitometry analysis of the signals was performed with NIH Image 1.60 software. The signal ratio *bcl-2*:*G3PDH* was then compared between samples.

Results

B lymphocytes from lymphocytotic sheep show an increase in *ex vivo* survival

BLV infection induces the expansion of the B cell population that is reflected by an increase of the B:T lymphocyte ratio. We thus designate sheep with an elevated number of circulating B cells and an increased B:T ratio as lymphocytotic. Table 1 shows that the B:T ratio was between 0·13 and 0·5 in normal sheep and reached 1·3 to 5 in lymphocytotic sheep. This inverted B:T ratio was accompanied by a seven- to 35-fold increase in the total number of circulating blood B lymphocytes that appeared histologically normal. Some BLV-infected sheep presented a seemingly normal B:T ratio although the total number of circulating B cells was slightly elevated (Table 1); nevertheless, based on their normal B:T ratio, these sheep were defined as asymptomatic. The haematological status of the sheep remained stable during the 9 month study and no sheep developed lymphosarcoma.

Table 1. B:T ratio, B lymphocyte counts and percentage of B lymphocytes in S phase in control and BLV-infected sheep

Sheep	B:T*	B cells per mm³†	B cells in S phase (%)‡
Controls			
128	0·13	188	0·04
190	0·2	151	0
126	0·5	289	0·15
Asymptomatic			
35¶	0·1	321	0·02
99¶	0·1	626	0·07
105 ‖	0·3	788	0·12
Lymphocytotic			
92¶	1	1860	0·36
120‖	1·3	1500	0·12
142‖	1·5	1807	0·38
85¶	2	1640	0·61
79¶	3·25	1726	0·3
121‖	5	7093	0·56

* The B:T ratio was estimated using double immunofluorescence labelling with the sheep anti-CD5 (ST-1A) and anti-CD21 (DU2-104) MAbs.
† The B cell counts were calculated from the percentage of CD21-positive cells among PBMC out of the total lymphocytes per mm³.
‡ Percentage of B cells in S phase was estimated using labelling of B cells with the CD21 MAb and incorporation of propidium iodide after cell permeabilization.
¶ Sheep infected for 2 years.
‖ Sheep infected for 4 years.

We initially assessed B lymphocyte survival in 24 h *ex vivo* PBMC cultures from uninfected and lymphocytotic sheep (sheep 128 and 121, respectively). B cell survival was estimated using double-fluorescence flow cytometry analysis; B cells were detected with an anti-CD21 antibody (DU2-104) that recognizes all the immunoglobulin-bearing lymphocytes in sheep (W. Hein, personal communication) and cell death was initially analysed with the TUNEL reaction. As illustrated in Fig. 1(*a*), normal B lymphocytes from *ex vivo* cultures showed extensive cell death (64%) and by contrast, B cells from the lymphocytotic sheep 121 presented only limited cell death (13%). We found that the total number of cells and the percentage of B lymphocytes were similar before and after the 24 h culture, indicating that the cell bodies and the antigenic structures were preserved despite the ongoing cell death (data not shown). Cell death in the B lymphocytes was specifically induced by the cell culture conditions as the TUNEL reaction was negative on freshly harvested B cells from uninfected and BLV-infected sheep (data not shown).

The TUNEL reaction identifies apoptotic cell death, as DNA fragmentation is the biochemical feature that best characterizes this type of death. However other criteria, such as loss of cell volume and chromatin condensation, have to be met in order definitely to characterize apoptosis (Darzynkiewicz *et al*., 1992). In the *ex vivo* cultured B cells, loss of cell volume was estimated using the light scatter profile by flow cytometry analysis: the apoptotic B cells appeared as a distinct population with a lower size (forward scatter axis; FCS) compared to the live B cells (Fig. 1*b*). Chromatin condensation was assessed using propidium iodide staining of the apoptotic B cell nuclei that appear as a hypodiploid population in FACS analysis (Fig. 1*c*). The percentages of apoptotic condensed nuclei in both the uninfected and lymphocytotic sheep cultured B lymphocytes were similar to the percentage of TUNEL-positive B cells and to the percentage of condensed B cells (Fig. 1). All these data demonstrate that *ex vivo* cell death in B cells relates to programmed cell death.

Resistance of B cells to apoptosis was further analysed in other *ex vivo* cultures of sheep PBMCs. Overall, BLV-infected B lymphocytes presented a higher level of survival (Fig. 2): cultures from normal sheep B lymphocytes showed 50 to 64% B cell death (mean 57·3 ± 5·7) whereas cultures from asymptomatic sheep showed 45 to 40% B cell death (mean 42 ± 2·2)

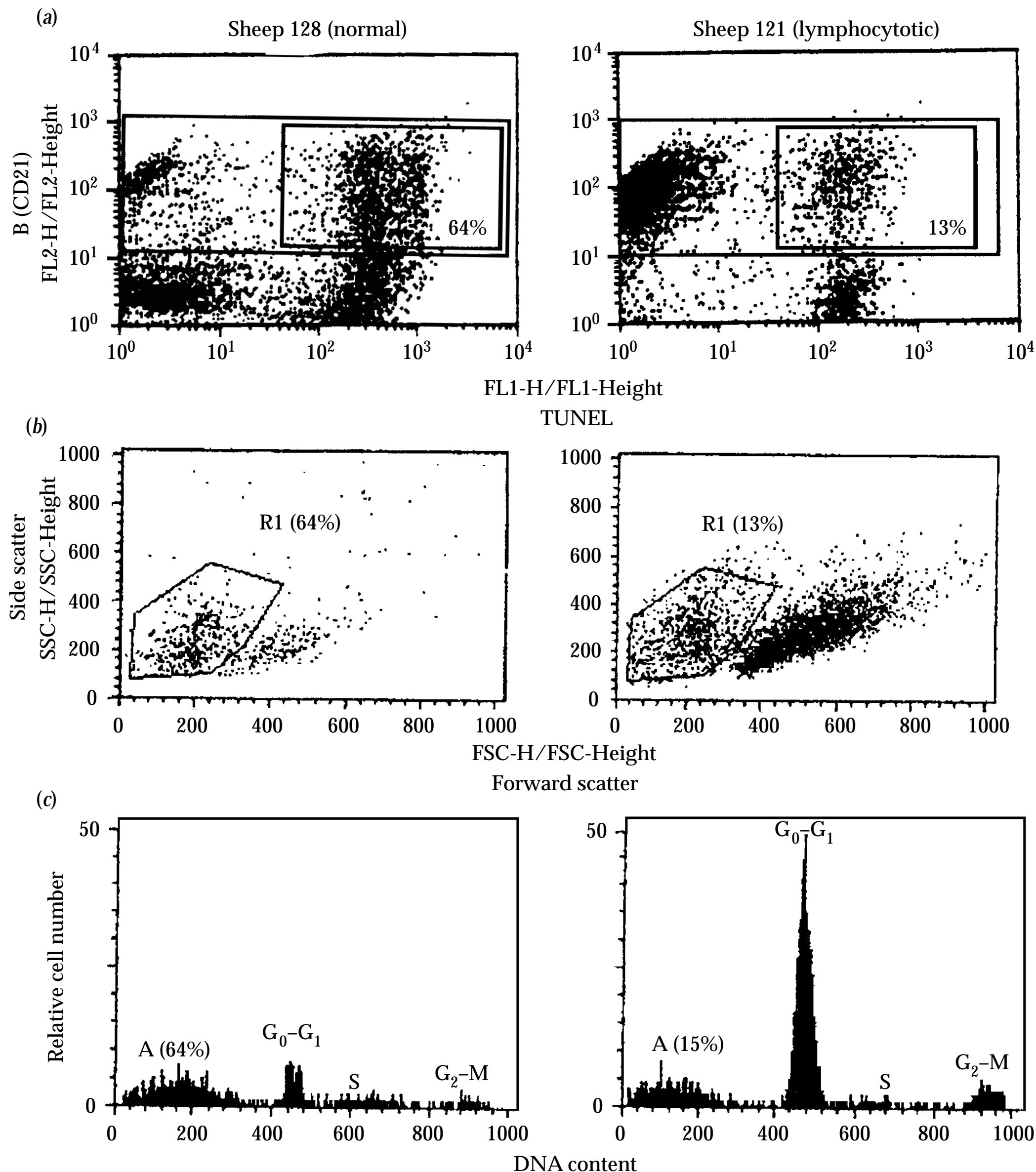

Fig. 1. *Ex vivo* apoptosis in 24 h cultured sheep PBMCs. PBMCs from sheep 128 (normal) and sheep 121 (lymphocytotic) were cultured for 24 h, labelled with the DU2-104 IgM MAb and processed for several analyses to detect apoptosis. (*a*) TUNEL reaction. The percentage cell death among the gated B cells is indicated. (*b*) Analysis of morphological modifications using light scattering properties; the regions of the smaller cells that are apoptotic dead cells are depicted. (*c*) Detection by propidium iodide staining of the apoptotic nuclei that appear as a hypodiploid subpopulation. The cells in the G_0–G_1, S and G_2–M phases are indicated. Cell doublets were excluded.

and cultures from lymphocytotic sheep showed 13 to 38% B cell death (mean $29 \pm 8{\cdot}2$). It thus appears that the extent of B lymphocyte survival correlated with the clinical status of the sheep (Fig. 2).

Analysis of the DNA content revealed that the *ex vivo* cultured B cells of the lymphocytotic sheep 121 appeared mainly in the G_0–G_1 phase (70%) with 5 and 9% of the B lymphocytes in the S and G_2–M phases, respectively; by contrast the *ex vivo* cultured B cells from the uninfected sheep 128 were mainly apoptotic (64%), 18% of the B cells were in the G_0–G_1 phase and 13 and 5% were in the S and G_2–M phase, respectively. Similar cell cycle distributions were obtained with two other uninfected sheep (185 and 126) and two other lymphocytotic sheep (79 and 85) (data not shown).

This finding indicates that the lymphocytotic B cells in *ex vivo* cultures accumulate in the G_0–G_1 phase, a feature associated with reduced apoptotic cell death. In addition, it was noticed that the percentage of cultured B cells in the S–G_2–M phase was not significantly different between uninfected and lymphocytotic sheep.

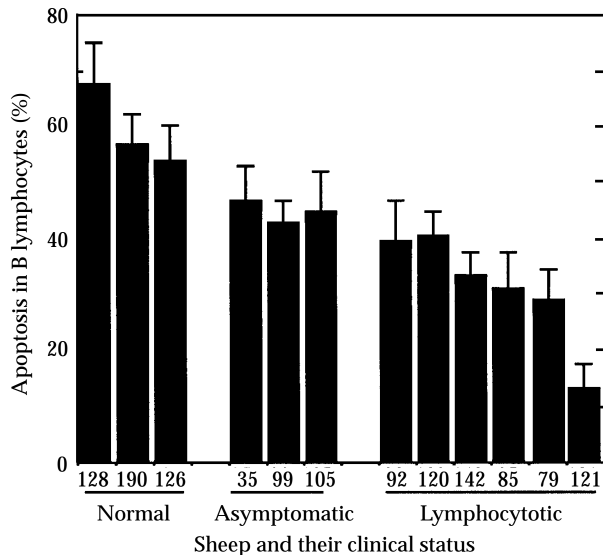

Fig. 2. *Ex vivo* percentage apoptosis in 24 h cultured sheep B lymphocytes measured using the TUNEL assay. The results are means and SD obtained from two experiments. Sheep are ordered according to increasing B:T ratio (Table 1) and their identification is reported with their clinical status. The high-powered Student-Newman-Keuls test (SAS Institute, 1990) confirmed that the differences in B cell survival between the normal, asymptomatic and lymphocytotic sheep were significant. Similar results of B cell death in PBMC cultures were obtained with detection of apoptosis using propidium iodide incorporation after cell permeabilization instead of the TUNEL reaction.

B cell survival *ex vivo* correlates with BLV expression

Several reports have mentioned that monolayer or cell suspension cultures from BLV-infected tissues presented cell degeneration concomitant to virus production (Van der Maaten *et al.*, 1974; Cornefert-Jensen *et al.*, 1969). However, whether virus replication in *ex vivo* PBMC cultures is associated with cell death is unknown. We thus aimed to determine whether the extended B cell survival described above could be found among B cells that replicate the virus or among reactive unproductive B cells.

BLV has been shown to be expressed in sheep B cells *in vitro* (Djilali *et al.*, 1987). Fig. 3(*a*) confirms that BLV CA capsid expression is confined to B cells in 24 h PBMC cultures. Simultaneous detection of intracellular CA production and cell death was performed on sheep PBMCs using double staining analysis by flow cytometry. The results obtained with the lymphocytotic sheep 79 (Fig. 3*b*) show that most CA-producing B cells (64%) resist apoptosis. Experiments with sheep 105, 120, 142 and 121 PBMCs revealed that overall $60{\cdot}6 \pm 5{\cdot}8$% of the CA synthesizing B cells survive after a 24 h culture.

Similar data were obtained with the virus Env protein, although its expression level was lower than for CA (data not shown). Unfortunately, we were unable to detect significant amounts of Tax and Rex production in any of the sheep PBMCs as the available antibodies (Willems *et al.*, 1990) gave a high background in control cells. In any event, detection of CA expression well reflects the production of other virus gene products including Tax and Rex.

CA production was assessed in B cells from the BLV-infected sheep and revealed that the extent of *ex vivo* B cell survival correlated with the percentage of CA-producing B cells (correlation coefficient $R^2 = 0{\cdot}949$) (Fig. 4). Altogether, these data strongly support the hypothesis that BLV infection and expression in B cells are associated with increased B lymphocyte survival. This finding argues for a role of virus gene expression in protection against apoptosis.

It could be suggested that virus production in the *ex vivo* PBMC cultures may trigger a specific immune stimulation in anti-BLV responsive B cells that would result in B cell survival and proliferation (Cherney *et al.*, 1994; Cambier & Ransom, 1987; Mager *et al.*, 1994). However, as seen in Fig. 1(*c*), no obvious difference in overall DNA synthesis could be observed between the 24 h cultures of B lymphocytes from uninfected and lymphocytotic sheep. Furthermore, [^{3}H]thymidine incorporation after 48 and 72 h culture did not reveal any significant difference between normal, asymptomatic and lymphocytotic PBMC cultures (data not shown): this indicates that BLV antigen production in these cultures does not induce a potent immune B cell response and does not favour the hypothesis that *ex vivo* B cell survival occurs through specific antigenic immune stimulation.

Increased B cell survival in BLV-infected lymphocytes is not accompanied by *bcl-2* transcriptional activation or increase in CD43 expression

Resistance to apoptosis in BLV-infected B cells could be associated with altered expression of cellular proteins involved in prolonged B cell survival. The field of death-modulating cellular factors is extending and comprises a great variety of proteins such as cytokines, proto-oncogenes, anti-oncogenes, cell cycle regulators and adhesion molecules. We focused on the study of two molecules important for survival in the B cell lineage, namely Bcl-2 and leukosialin (CD43).

Modulation of Bcl-2 expression is a determinant of life and death in B lymphocytes (Katsumata *et al.*, 1992). For instance, there is a correlation between the levels of *bcl-2* mRNA expression and the longevity of B-lineage cells (Haury *et al.*, 1993), and B lymphocytes from *bcl-2* transgenic mice have an extended life span *ex vivo* (Katsumata *et al.*, 1992). We thus measured *bcl-2* expression in highly purified sheep B cells, as the expression in other leukocyte populations may mask any modulation of *bcl-2* expression in B cells. Detection of Bcl-2 protein *in situ* was not possible due to the lack of antibody against the sheep protein. We performed semi-quantitative RT–PCR on RNA extracts from purified B cells with oligonucleotides that recognize the *bcl-2* gene from different species. The *bcl-2* mRNA level was similar in B cell extracts from both normal and lymphocytotic sheep (Fig. 5). In conclusion, it does not appear that *bcl-2* transcription is altered in B cells from lymphocytotic sheep.

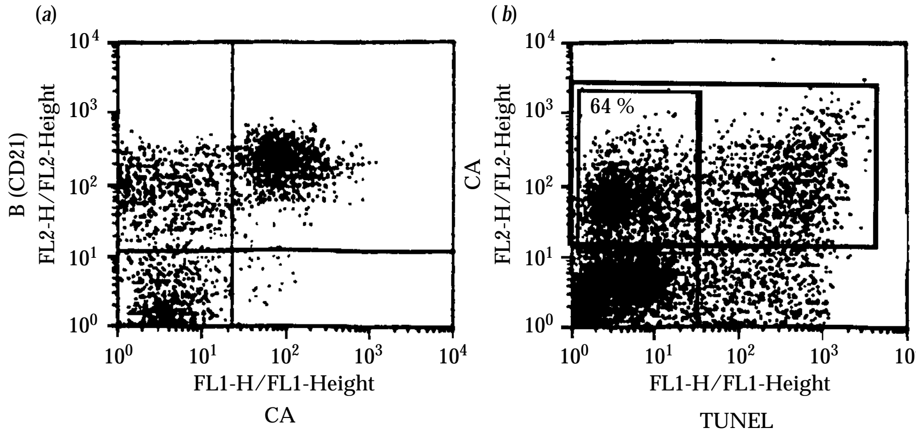

Fig. 3. (*a*) Production of CA in *ex vivo* B lymphocytes from sheep 79 as an example. Sheep 79 PBMCs were cultured for 24 h, labelled with DU2-104 revealed by PE-conjugated anti-mouse IgM (FL2 axis), then permeabilized and labelled with anti-CA MAbs revealed by FITC-conjugated anti-mouse IgG1 (FL1 axis). (*b*) Apoptosis and CA production in sheep 79 PBMCs. Permeabilized sheep 79 PBMCs were labelled for CA detection (FL2 axis) and TUNEL reaction (FL1 axis). The percentage of surviving cells among the gated CA-producing cells was 64%.

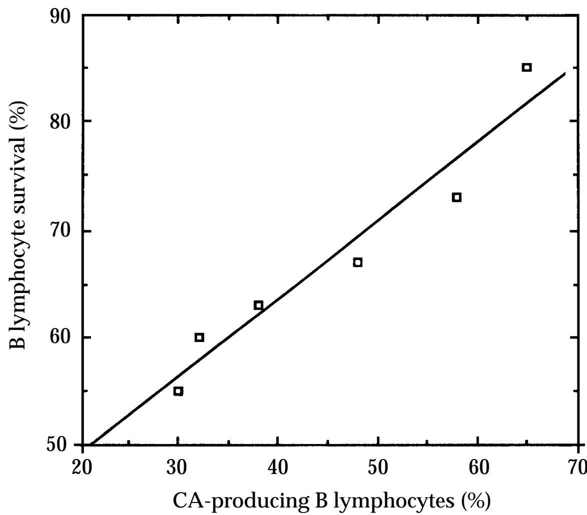

Fig. 4. Correlation between CA production and sheep B cell survival *ex vivo*. A regression analysis relating the percentage B cell survival to the percentage of B cells expressing CA demonstrates a close correlation between the number of CA-producing B cells and B cell survival ($R^2 = 0{\cdot}949$).

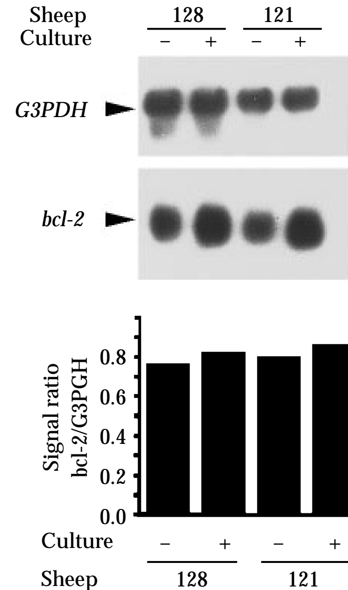

Fig. 5. Expression of *bcl-2* mRNA in purified B cells from a normal and a lymphocytotic sheep. RT–PCR for detection of *G3PDH* and *bcl-2* mRNA expression in 10^4 normal sheep purified B cells (128) and lymphocytotic sheep purified B cells (121) before and after 24 h culture. The signal ratio of the densitometry values *bcl-2*:*G3PDH* is shown.

B lymphocytes from transgenic mice for the leukosialin molecule (CD43) display an extended survival in *ex vivo* cultures (Dragone *et al.*, 1995). CD43 has a unique developmental programme in mouse B cells: it is expressed on early B cell progenitors, terminally differentiated plasma cells and on the majority of B1 cells (Wells *et al.*, 1994). In the mouse, B1 cells represent an independent B cell lineage and are characterized by their anatomical localization, function and expression of markers not found on conventional B cells, such as CD5 and/or CD11b (Haughton *et al.*, 1993). As BLV infection in sheep is characterized by an expansion of $CD11b^+$ B lymphocytes, we thus tested whether CD43 expression was specific to the expanding B cell population. Double immunostaining with an anti-sheep CD43 antibody and DU2-104 was performed on the PBMCs from three control and three lymphocytotic sheep. Surprisingly, almost all (over 99%) normal circulating sheep B cells expressed CD43, a finding that contrasts with the absence of CD43 on most peripheral blood B cells in mouse and human (Fig. 6). In addition, it was found that the level of CD43 expression was unchanged in lymphocytotic sheep B cells compared to controls (Fig. 6).

In conclusion, neither *bcl-2* transcription nor CD43 protein expression was altered in lymphocytotic BLV-infected sheep.

Persistent lymphocytosis is both a lymphoaccumulative and lymphoproliferative syndrome

So far, our data support the idea that BLV-induced B lymphocyte expansion results from enhanced B cell survival.

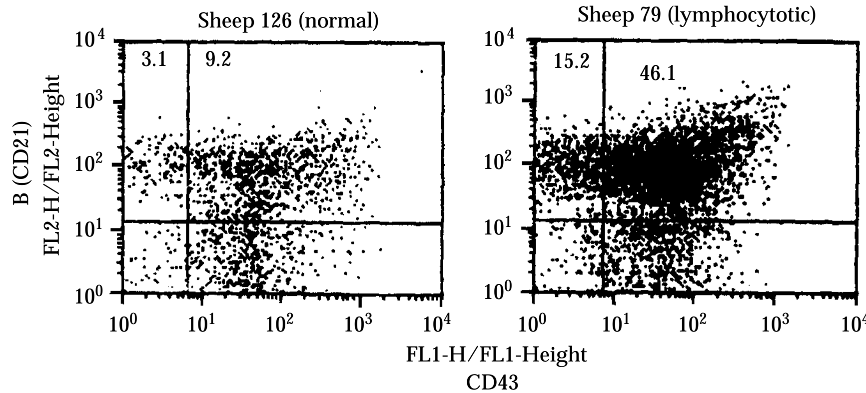

Fig. 6. CD43 expression on normal sheep 126 B cells (*a*) and lymphocytotic sheep 79 B cells (*b*). Double staining was performed using the anti-B cell DU2-104 antibody (FL-2) and the anti-CD43 Co-44B8 (FL-1). Numbers in quadrants indicate the percentage of the selected population relative to the total PBMCs.

As such altered homeostatic regulation in a cell population can result from decreased cell death and/or increased cell proliferation, we analysed cell cycling among directly isolated sheep B cells without any culture step. Table 1 shows that the percentage of B lymphocytes in S phase was similar in normal and BLV-infected asymptomatic sheep, and varied between 0 and 0·12% (mean 0·06 ± 0·058%). However, the percentage of B cells in S phase was slightly increased in lymphocytotic sheep and reached 0·12 to 0·61% (mean 0·33 ± 0·2%). A fivefold increase in the *in vivo* B cell proliferation probably participates in BLV-induced lymphocytosis.

These cell cycle data obtained with freshly isolated B cells are quite different from those obtained with cultured B cells. As shown in Fig. 1(*c*), the *ex vivo* culture of uninfected or lymphocytotic B cells resulted in a potent stimulation of DNA synthesis with over 10% of cycling B cells; the *ex vivo* culture probably induced a multiplicity of signals leading to cell proliferation, which has also been found with T cells. In this case, no significant differences in the percentage of cycling B cells could be observed between control and lymphocytotic sheep cultured B cells.

In any event, the slightly increased proportion of B cells in S phase was consistently found in the freshly harvested B cells from lymphocytotic sheep compared to uninfected sheep. Assuming that the cell cycle activity measured in our experiment reflects the *in vivo* proliferative activity, one can propose that the increased proportion of cycling B cells yields more B cells that will further divide and that will eventually lead to cellular accumulation. Overall, our results show evidence for an association between extended B cell survival and increase in B cell cycling in BLV-induced B cell expansion.

Discussion

Some viruses can prolong the life-span of the cells they infect and thus facilitate their own replication, spread and persistence. Virus proteins known to favour cell survival act in different ways: for instance, the simian virus 40 T antigen and the papillomavirus E6 protein modulate p53 function (Symond *et al*., 1994; Pan & Griep, 1994), the cowpox virus CrmA protein inactivates the cysteine protease interleukin 1β-converting enzyme (Gagliardini *et al*., 1994), the LMP protein of Epstein–Barr virus (EBV) upregulates *bcl-2* expression (Henderson *et al*., 1991) and the BHRF1 protein of EBV has similarities to Bcl-2 and functions in an analogous manner (Henderson *et al*., 1993). By contrast, studies on retroviruses have mainly shown their pro-apoptotic properties. Indeed lentiviruses such as human immunodeficiency virus (Groux *et al*., 1992) and feline immunodeficiency virus (Ohno *et al*., 1993) and oncoviruses such as feline leukaemia virus type C (Rojko *et al*., 1992) and a mutant Moloney virus (Saha *et al*., 1994) have been associated with cell death induction. In the case of HTLV-1, several reports argue for its involvement in induction of apoptosis: in the spinal cord of patients with HTLV-1-associated myelopathy, many CD45RO T lymphocytes show signs of apoptosis (Umehara *et al*., 1994); some HTLV-1 variants that induce lethal leukaemia in rabbits have been shown to induce apoptosis in lymphoid cells both *in vitro* and *in vivo* (Leno *et al*., 1995). However, HTLV-1 infection of normal PBMCs *in vitro* can generate long-lived T cell cultures, thus indicating that HTLV-1 somehow promotes cell survival (Persaud *et al*., 1995). By contrast, very few reports mention that HTLV-1 may be involved in protection against apoptosis (Copeland *et al*., 1994).

We report here that B lymphocytes from BLV-infected sheep show an increased survival potential *ex vivo* that correlates with clinical status and virus production. Several hypothetical mechanisms can be proposed to explain the BLV-associated increase in cell survival. First, specific antigenic stimulation has been shown to rescue B cells from entering apoptosis and to induce their proliferation in the presence of T cells (Cherney *et al*., 1994; Cambier & Ransom, 1987). We found that the *ex vivo* BLV product synthesis was not accompanied by a significant increase in PBMC proliferation compared to normal sheep PBMCs: this observation does not favour the involvement of a specific antigenic response in the B cell survival phenomenon.

The increase in B cell survival could also result from an expansion of a cell population with an enhanced capacity for survival. In a previous study, we reported that the expanding

B cell population in sheep always carries the CD11b molecule but not the CD5 molecule, in contrast to cattle (Schwartz *et al.*, 1994). Other authors also described that the CD5 molecule is not consistently expressed on lymphocytotic and leukaemic sheep B cells (Birkebak *et al.*, 1994; Murakami *et al.*, 1994). In any event in the mouse, the CD11b marker, associated or not with the CD5 molecule, is found on a subpopulation of B cells named the B1 lymphocytes (Arnold *et al.*, 1994). The mouse B1 lymphocytes are capable of self renewal and they show a prolonged survival *in vitro* (Haughton *et al.*, 1993). It is thus possible that resistance to cell death in BLV-infected B cells relates to their 'B1-like phenotype' reflected by CD11b expression at the cell surface. CD11b is mainly expressed on myeloid cells, is found associated with the CD18 molecule and functions as an adhesion complex that interacts with several cell surface and soluble ligands including intercellular adhesion molecule-1, fibrinogen, iC3b, factor X and heparin (Diamond *et al.*, 1995); however, its role in cell survival has not been documented.

Another mechanism by which BLV infection prolongs B cell life-span *ex vivo* could be through altering the expression of cellular genes involved in cell survival. Although regulation of *bcl-2* transcription has been implicated in many aspects of lymphocyte life (Haury *et al.*, 1993; Vaux *et al.*, 1988; McDonnell *et al.*, 1989), we could not find altered expression of *bcl-2* mRNA in BLV-infected lymphocytotic B cells compared to normal B cells. There are circumstances in which the capacity for B cell survival is independent of *bcl-2* expression. In that respect, B1 cells are known to be long-lived both *in vivo* and *in vitro* and they present a very reduced *bcl-2* mRNA level compared to conventional B cells (Haury *et al.*, 1993), suggesting that other active molecules promote their survival. The B cells from CD43 transgenic mice show extended *ex vivo* survival (Dragone *et al.*, 1995), suggesting that CD43 may play a role in the extended life-span of B1 cells that generally express this molecule. This hypothesis encouraged us to test for CD43 expression on B lymphocytes from BLV lymphocytotic sheep. However, no change in CD43 expression could be found between normal and BLV-infected sheep B cells and notably quite high levels of CD43 were found on normal circulating sheep B lymphocytes. This is in contrast with human and mouse B cells that express CD43 at very specific stages of B cell differentiation (Wells *et al.*, 1994). The significance of CD43 expression on sheep B cells is unknown; immune response in sheep and cattle often seems to involve B1 cells and γ/δ T lymphocytes, both types of cells being considered as primitive immune cells; CD43 expression on B cells may play an important role in the ruminant immunity.

BLV expression per se could be involved in B cell resistance to death. Actually, we observed a linear correlation between the level of CA expression and the extent of B cell survival *ex vivo*, suggesting that expression of some virus gene may promote B cell survival. In HTLV-1-induced immortalization *in vitro*, T cell transformation is associated with high levels of CA expression (Persaud *et al.*, 1995) and the level of resistance to Apo1-induced apoptosis in adult T cell leukaemia-derived lines correlates with CA production (Copeland *et al.*, 1994): this indicates that virus gene expression may well be associated with cell survival. CA production reflects expression of many other virus proteins that could be responsible for resistance to cell death. *In vitro*, we found that BLV Tax expression confers resistance to cell density (I. Schwartz-Cornil, D. Le Rhun, I. Oswald, V. Lainé, L. Willems, B. Schwartz and D. Levy, unpublished results), suggesting that in some circumstances, BLV Tax plays a role in B cell survival. Other virus gene products may also be involved, such as GIV, whose level of expression has been found to be increased in persistent lymphocytosis (Alexandersen *et al.*, 1993). However, it should be kept in mind that BLV is very weakly expressed *in vivo* and the way viral products may directly affect B cell survival *in vivo* is unclear; in that respect, several hypotheses can be proposed: viral expression may occur in a specific but uncharacterized tissue compartment, it may be produced at an undetectable level or during a short period of time but is anyhow sufficient to affect B cell survival.

In addition to B cell survival, we assessed the involvement of B cell proliferation in BLV-induced lymphocytosis. Cell cycle analysis revealed that lymphocytosis was accompanied by a slight increase in the percentage of B cells in the S phase (0·33% as an average versus 0·06% in normal sheep). Although this increase may participate in the expansion of the B lymphocyte population, it also indicates that the vast majority of circulating B cells in persistent lymphocytosis are resting B cells in the G_0–G_1 phase of the cell cycle. This finding is in contrast with a report showing quite elevated percentages (15%) of cycling B cells in lymphocytotic cattle *in vivo* (Matheise *et al.*, 1992). The difference in cell cycling *in vivo* between infected sheep and cattle may reflect a more important involvement of cell proliferation in the pathogenesis of lymphocytosis in cattle.

In conclusion, our data provide evidence that the oncovirus BLV alters the B cell population homeostasis with both an increase in B cell cycling and in B cell resistance to apoptosis. This dual phenomenon encountered in the preleukaemic stages of the disease probably participates in the pathogenesis of the BLV-induced lymphocytic syndrome. This leads the way for further investigations to delineate the involvement of viral and cellular genes in the altered regulation of B cell death and proliferation associated with BLV infection.

We are grateful to W. Hein for the generous gift of the DU2-104 hybridoma and D. Portetelle for the anti-CA, -Env, -Tax and -Rex antibodies. We thank A. Dialo and P. Dreyfus for their useful advice and F. Crespeau for the easy access to FACS analyses. We are especially indebted to B. Schwartz and P. De Sepulveda for their helpful critical comments on the manuscript, to M. Benardi, M. Saana and P. Leascoat for statistical analyses and to B. Polack for his computer expertise. A.M. was supported by the Spanish Directorate for Science and Technology, project PB93-0160-CO-02.

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Received 22 May 1996; Accepted 30 August 1996