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Research paper

Host soluble factors that regulate the synthesis of the major core protein of the bovine leukemia virus (BLV) in a naturally infected neoplastic B-cell line

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ABSTRACT

Bovine leukemia virus (BLV) is a B-cell tropic *Deltaretrovirus* that induces a lifelong infection and causes a fatal lymphosarcoma in less than 10% of the infected cattle. BLV is usually present in its host in a transcriptional repressed state but becomes de-repressed a few hours after the infected lymphocytes are cultured *in vitro*. In the present study we have examined the effect of soluble host factors and various substances on the synthesis of the major BLV protein (p24) in a permanent culture (cell line NBC-10) of neoplastic B-lymphocytes derived from BLV-infected cattle. Certain batches of fetal calf serum (FCS) and bovine platelet lysates (PLy) induced a rapid and drastic increase of the synthesis of BLVp24 in the NBC-10 cells. Neutralization experiments with specific antibodies demonstrated that the transforming growth factor-β (TGF-β) was responsible for the stimulatory activity of FCS and PLy on the synthesis of BLVp24 in the NBC-10 cells. Recombinant TGF-β also stimulated the synthesis of BLVp24 in cultures of peripheral blood mononuclear cells (PBMCs) obtained from BLV-infected cattle. Mitogens, phorbol-myristate-acetate and prostaglandin E_2, previously shown to stimulate the expression of BLV in cultures of PBMC, did not induce the synthesis of BLVp24 in cultures of NBC-10 cells. Plasma, serum and milk from BLV-negative cattle inhibited the synthesis of BLVp24 induced by FCS, PLy or TGF-β in the NBC-10 cells. The blocking activity was found in the whey and the β-casein fractions of bovine milk. The relevance of these findings with regard to the previously reported plasma factor (PBB) with blocking activity on the expression of BLV in short-term PBMC cultures is discussed. Based on the information obtained in the present study we have standardized a reproducible and rapid assay system for the identification of factors that regulate the synthesis of BLVp24 in naturally infected neoplastic B cells.

Abbreviations: BLV, bovine leukemia virus; BLVp24, major core BLV protein; FCS, fetal calf serum; hr, human recombinant; HTLV, human T-cell leukemia virus; IGF, insulin-like growth factor; LTR, long terminal repeat; MTT, methylthiazoltetrazolium; OD, optical density; PAF, platelet activating factor; PBB, plasma BLV-blocking factor; PBMC, peripheral blood mononuclear cells; PD-ECGF, platelet-derived endothelial cell growth factor; PDGF, platelet-derived growth factor; PLy, platelet lysates; RANTES, regulated on activation, normal T cell expressed and secreted; S-MEM, minimal essential medium modified for suspension cultures; TGF, transforming growth factor; TNF, tumor necrosis factor.

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1. Introduction

Bovine leukemia virus (BLV) is the etiologic agent of enzootic bovine leukemia, a fatal chronic lymphoproliferative neoplastic disease. The majority of cattle infected with BLV remain clinically asymptomatic throughout their life. Up to approximately one-third of infected cattle develop a persistent lymphocytosis, an essentially benign condition characterized by a permanent expansion of the B-cell population in peripheral blood. Despite the fact that BLV infection persists for the life of the animal, less than 10% of the infected cattle develop enzootic bovine leukemia (lymphosarcoma) (Ferrer et al., 1979; Ferrer, 1980).

BLV infection is prevalent in several regions of the world. It has been estimated that more than 70% of the dairy herds in the United States contain infected cattle (Ferrer, 1980; NAHMS, 1996). BLV infection is also prevalent among dairy herds in Argentina (Ghezzi et al., 1997; Trono et al., 2001). The economic impact of BLV infection is mainly due to the loss of cattle with lymphosarcoma, a fatal neoplastic disease, and to the loss of exports markets, as many countries have implemented strict measures to prevent the importation of infected cattle as well as of semen and embryos from these animals (reviewed in Ferrer, 1980).

BLV is a B-cell tropic virus closely related to the human T-cell leukemia viruses (HTLV-I and -II). Expression of BLV is thought to be blocked at the transcriptional level *in vivo*, since neither viral particles or proteins, nor viral RNA has been readily detected in freshly isolated peripheral blood lymphocytes or tumor cells (Stock and Ferrer, 1972; Baliga and Ferrer, 1977; Kettmann et al., 1980, 1982; Gupta et al., 1984; Van den Broeke et al., 1988). However, a few hours after these cells are cultured *in vitro* they begin to synthesize virus particles (Stock and Ferrer, 1972, Baliga and Ferrer, 1977), viral antigens (Gupta and Ferrer, 1982; Gupta et al., 1984) and viral RNA (Gupta et al., 1984; Zandomeni et al., 1992). The addition of fetal calf serum (FCS) (Zandomeni et al., 1992) to the culture medium greatly stimulates viral expression in short-term cultures of peripheral blood mononuclear cells (PBMCs) from BLV-infected cattle. Since this stimulation is not associated with an increase in viral DNA synthesis (Zandomeni et al., 1992), it seems clear that the detection of viral products in 24-h cultures of PBMC is the result of the de-repression of the viral genome rather than of the expansion of the subpopulation of infected lymphocytes. The factor(s) responsible for the stimulating effect of FCS on the expression of BLV in these cultures has not been identified. Expression of BLV in PBMC cultures is also increased by the addition of platelet lysates (PLy) (Tsukiyama et al., 1987), and mitogens such as PHA (Miller et al., 1969; Stock and Ferrer, 1972; Baliga and Ferrer, 1977; Chatterjee et al., 1985; Djilali et al., 1987; Jensen et al., 1990; Kidd and Radke, 1996), ConA (Driscoll et al., 1977; Lagarias and Radke, 1989) and bacterial LPS (Jensen et al., 1992; Kidd and Radke, 1996).

The addition of plasma from cattle and humans as well as of bovine lymphatic fluid blocks BLV expression in short-term cultures of PBMC from infected cattle (Gupta and Ferrer, 1982; Gupta et al., 1984; Zandomeni et al., 1992, 1994). It has been postulated that these fluids contain a factor, termed PBB (plasma BLV-blocking factor), whose activity accounts for the repressed state in which BLV, like its relative, HTLV, is present in its natural host. The available data indicate that the PBB factor is neither an antibody nor interferon (Gupta and Ferrer, 1982; Chatterjee et al., 1985). Recently, a fibronectin-containing complex isolated from bovine plasma has been shown to inhibit the synthesis of BLVp24 in short-term PBMC cultures (van den Heuvel et al., 2005).

Previous studies (Ferrer et al., 1971, 1973) have shown that the synthesis of BLV antigens in permanent cultures of naturally infected neoplastic lymphoid cells derived from cows with histologically confirmed lymphosarcoma (Marshak et al., 1963; Hare et al., 1968) is highly influenced by the culture conditions. In the present study we have identified the conditions and factors that influence the synthesis of the major core BLV protein (p24) in one of these cultures, termed cell line NBC-10. Based upon the information obtained we have used the NBC-10 cells as the indicator system to develop a reliable and practical assay for the detection of host soluble factors with regulatory activity on the synthesis of BLVp24. We show that the stimulatory activity of FCS and PLy on the expression of BLV in the NBC-10 cells is due mainly to the transforming growth factor beta (TGF-β). We also show that, in addition to plasma, blood serum, milk, milk serum (whey) and β-casein from BLV-negative cattle strongly inhibit the expression of BLV in the NBC-10 cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The origin and characteristics of the cell line NBC-10 has been described (Marshak et al., 1963; Hare et al., 1968; Ferrer et al., 1971, 1973). The NBC cell lines were initially established and maintained in McCoy's 5A medium supplemented with heat-inactivated horse serum. They were adapted to grow in minimum essential medium modified for suspension cultures (S-MEM) supplemented with 5–10% of FCS (Bioser, GEN S.A.), 100 IU ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin. In our initial experiments we found that the NBC-10 cells grown in the presence of certain batches of FCS synthesize either very low or not detectable amounts of BLV p24. Thus, for the purpose of using the NBC-10 cells as the indicator system in an assay to identify factors that stimulate the expression of BLV, stock cultures of NBC-10 cells were routinely maintained as stationary cultures in polystyrene culture bottles in the presence of 10% FCS from these batches.

2.2. Molecular characterization of NBC-10 cell line

A PCR was carried out to determine if V, D and J genes of the bovine immunoglobulin heavy chain (IgH) locus were rearranged or not in the NBC cell lines. DNA was organically extracted from cell pellets as described (Abbott et al., 1988). Amplification was carried out on a Programmable Thermal Controller model PTC-100 (MJ Research

Inc.). Primer VHF (ccc tcc tct ttg tgc tgt c) anneals to conserved regions of the VH leader and primer FR3 rev (ttg tcc ttg gtg atg ctg ag) anneals to the third framework portion of the V region of the IgH locus. Primers IR 0.5 for (caa cag ccc tct ctc ctc) and IR1 rev (cct ggt cct gct caa gtc a) anneal to conserved regions of the JH locus (Verma, 2007). Amplification was carried out with primers VHF and IR1 rev at a final concentration of 0.5 μM and 1 μg of genomic DNA in a reaction buffer containing 2 mM magnesium chloride, 200 μM each dNTP and 0.8 U Taq polymerase. After initial denaturation at 94 °C for 2 min, reactions were cycled 36 times through 94 °C (1 min), 64 °C (40 s) and 72 °C (1 min) with a final extension step of 5 min at 72 °C. PCR products were resolved on a 1.2% agarose gel and visualized under ultraviolet light after ethidium bromide staining. DNA extracted from spleen cells was used as positive control while negative controls included DNA extracted from the cell line MDBK and the reaction mixture without the addition of the template DNA.

2.3. Phenotypic analysis of NBC-10 cell line

The expression of IgM at the cell surface was determined in NBC-10 cells by flow cytometry after labeling the cells with a mouse anti-bovine IgM mAb (cell line BiG73A, VMRD) and using a goat anti-mouse IgG (specific for the Fc gamma fragment) conjugated with R-Phycoerythrin as secondary Ab. PBMC from a BLV-infected cow with persistent lymphocytosis were used as positive control. Controls for autofluorescence (cells not labeled with antibodies) and for non-specific binding of the PE-conjugated Ab were also included. After being incubated with the mentioned Abs, the cells were fixed in 0.2% buffered formaldehyde and analyzed using a FACS flow cytometer (FACSCanto, Becton Dickinson). Dot plots were performed using the Windmi® software.

2.4. Determination of the BLV status of the cattle used

Serum, plasma or milk samples were tested for BLV antibodies applying a highly sensitive and specific ELISA, designated ELISA 108. Samples obtained from cattle used as donor of plasma, serum or milk were negative in the ELISA 108 in two samples obtained with an interval of 1 month. These cattle were also negative in a PCR analysis of DNA extracted from peripheral blood leukocytes. A detailed description and evaluation of the ELISA 108 and PCR assay have been reported (Gutierrez et al., 2001).

2.5. Isolation and culture of PBMC

Peripheral blood was obtained from BLV-infected cows with persistent lymphocytosis. The procedure described by Zandomeni et al. (1992) with slight modifications was applied for this purpose. Briefly, blood was obtained by jugular venipuncture, using sodium citrate/EDTA (0.146 M Na citrate dihydrate, 0.1% EDTA, pH adjusted to 6.5 with citric acid) as anticoagulant. The buffy coat obtained after centrifugation ($1200 \times g$ for 20 min) of the blood was suspended in S-MEM containing sodium citrate and EDTA. The mononuclear cells were separated by centrifugation ($1300 \times g$, 20 min) through an Isolymph® gradient. The cells at the interface were suspended in S-MEM containing 20% autologous plasma and sodium citrate/EDTA and centrifuged at $600 \times g$ for 5 min. The resulting cell pellet was washed with S-MEM containing 5% autologous plasma, and then with S-MEM containing sodium citrate and EDTA. After centrifugation, the cells were suspended in S-MEM containing sodium citrate and EDTA and counted. Cell viability was determined using acridine orange ethidium bromide solution.

PBMCs were suspended in S-MEM at 6×10^6 cells ml^{-1} and cultured in 24-well polystyrene plates. To avoid the adherence of the cells to the plate, the surface of the wells was previously coated with a solution of 1% agarose in PBS. Cultures were maintained for 20–24 h at 37 °C and 5% CO_2 in horizontal agitation (18 rpm).

2.6. Methylthiazoltetrazolium (MTT) proliferation assay

The reduction of MTT to formazan by mithocondrial dehydrogenases was used as indicator of the number of metabolically active cells in the cultures. The procedure was carried out as follows: NBC-10 cell cultures were established in RPMI culture medium without phenol red at a concentration of 1.7×10^6 cells ml^{-1} in 96-well culture plates (volume of cultures = 150 μl). Cultures were set in quadruplicate: in three of them the reduction of MTT was quantified as described below, the fourth culture was used to determine the cell concentration and viability by direct count of the cells mixed with an acridine orange ethidium bromide solution (McGahon et al., 1995). The cultures were maintained at 37 °C in 5% CO_2 for 24–48 h. MTT (5 mg ml^{-1}) was added (10 μl per well) and the cultures were incubated for 4 h at 37 °C in 5% CO_2. Insoluble formazan was dissolved by adding 100 μl/well of a solution containing 20% SDS–50% dimethylformamide. After an overnight incubation of the plates at 37 °C in 5% CO_2, optical density (OD) at 570 nm was registered with an automated microplate reader. Controls were included in each experiment to assess the absence of reduction of MTT by the reactants included in the cultures (i.e. the culture medium, FCS, etc.). These controls consisted of replicates of the cultures where the cell suspension was replaced by an equal volume of culture medium.

2.7. Detection of the major core BLV protein (BLVp24) in cell extracts

Cell cultures were harvested and cell extracts prepared as described (Zandomeni et al., 1992). Total protein concentration in cell extracts was determined as described (Bradford, 1976) using the Bio-Rad dye reagent. BSA was used as the standard.

The capture ELISA used to quantify the presence of the BLVp24 core protein in cell extracts was essentially as described (Zandomeni et al., 1994) with some modifications. Briefly, the antigen was captured in 96-well microplates (Nunc Immuno Plate, MaxiSorp surface) by a bovine polyclonal serum, termed LFS 108, with high titer of anti-BLVp24 antibodies obtained from a cow with lymphosarcoma. Appropriate dilutions (1:5 to 1:50) of cell

extracts were applied to duplicate coated wells and incubated for 1 h at 37 °C. After the plates were washed, captured BLVp24 was detected by the addition of an anti-BLVp24 rabbit antiserum. This antiserum was prepared by repeated injections of BLVp24 purified to homogeneity as described (McDonald and Ferrer, 1976). The binding of the rabbit antiserum was revealed by the addition of a biotin-conjugated goat anti-rabbit IgG and peroxidase-conjugated streptavidin. OD at 450 nm was measured after the plates were incubated for 30 min with 3,3′,5,5′-tetramethyl benzidine and color developed. Duplicate wells in which the cell extracts were omitted were used as blanks. Each plate included a standard control curve with known amounts of BLVp24.

The mean amount of BLVp24 was calculated for each cell extract and results were expressed in nanograms of BLVp24 per milligram of total protein. The limit of detection of the capture ELISA was 11 ng ml^{-1}.

2.8. Detection of stimulatory and inhibitory activity of various fluids and substances on the synthesis of BLVp24

NBC-10 cells from stock cultures or PBMC were grown in 24-well culture plates at an initial cell density of 1.7 and 6×10^6 cells ml^{-1}, respectively (final volume of 1 ml). The plates were incubated for 20–24 h at 37 °C with 5% CO_2. Fluids and substances tested for stimulatory or blocking activity were added at the onset of the cultures. Each experiment included as controls: cells cultured without FCS (negative control) and cells cultured with 10% of FCS from a batch (e.g. batch no. 9861) having stimulatory activity on the synthesis of BLVp24 (positive control). Cultures supplemented with 10% of FCS from a batch not having stimulatory activity on the synthesis of BLVp24 were included as an additional control in the first series of experiments. They were omitted in some experiments to avoid the manipulation of an excessive number of cultures. When blocking activity was investigated, cultures supplemented with 10% of FCS with stimulatory activity and bovine plasma (or another fluid) with known blocking activity were included. All cultures were carried out in duplicate and cell viability was always determined at the end of the culture. Results are expressed as percentage of the concentration of BLVp24 obtained in the positive control. Percentage of control (% control) was calculated as: % control = $(A - B) \times 100/(C - B)$, where A = concentration of BLVp24 in extracts of NBC-10 cells cultured with 10% of FCS with stimulatory activity and plasma or another fluid in which blocking activity was investigated; B = concentration of BLVp24 in extracts of NBC-10 cells cultured without FCS (negative control); and C = concentration of BLVp24 in cellular extracts corresponding to the positive control cultures. The amount of BLVp24 synthesized in the stock cultures from which NBC-10 cells were taken was verified in each experiment.

2.9. Serum from bovine fetuses

Blood was collected by cardiac puncture from 15 fresh fetuses obtained at a local slaughterhouse. After the blood was centrifuged (1300 × g, 20 min at 10 °C), the serum was harvested and aliquots were stored at −20 °C. The gestational age of the fetuses was determined by the formula: $l = x(x + 2)$, where "l" represents the length of the fetus (from the foramen occipitale magnum to the base of the tail) measured in centimeters, and "x" is the gestational age expressed in months (Holy, 1983). Sera were heated at 70 °C for 30 min before being added to the cultures.

2.10. Substances tested for activity on the synthesis of BLVp24

ConA, pokeweed mitogen, *E. coli* LPS (L2654) and phorbol 12-myristate 13-acetate were all obtained from Sigma. PHA was obtained from Pharmacia. Progesterone, prostaglandin E_2, dexametasone and 17-β estradiol (Sigma) were dissolved in ethanol at 1 mg ml^{-1}; further dilutions were made in S-MEM. Human recombinant (hr) IL 1-α, hrIL 1-β, hrIL 2, hrIL 6, tumor necrosis factor alpha (hrTNF-α) were all obtained from Promega, and hrIL 10 from Chemicon. Human recombinant platelet derived growth factor (hrPDGF), the three isoforms of the transforming growth factor beta, hrTGF-β_1, hrTGF-β_2 and hrTGF-β_3, hr platelet-derived endothelial cell growth factor (hrPD-ECGF), hrTGF-α, hrRANTES (regulated on activation, normal T cell expressed and secreted), platelet activating factor (PAF) and insulin-like growth factor II (hrIGF-II) were all obtained from Sigma. Platelet factor 4, thrombospondin and β-thromboglobulin purified from human platelets were obtained from Calbiochem. The range of concentration at which these substances were tested is given in Tables 1 and 2.

Recombinant human activin A (Research Diagnostics) was tested at concentrations from 1 to 250 ng ml^{-1}. Inhibin purified from porcine ovary (Sigma) was tested at various concentrations between 50 pg ml^{-1} and 250 ng ml^{-1}.

Table 1
Activity of various substances on the synthesis of BLVp24 in cultures of NBC-10 cells.

Substances tested	Dose range	Stimulatory activity
Mitogens		
Phytohemagglutinin	20–200 μg ml^{-1}	No
Concanavalin A	1–200 μg ml^{-1}	No
Pokeweed mytogen	2–500 μg ml^{-1}	No
E. coli LPS	5–1000 μg ml^{-1}	No
Hormones		
Progesterone	0.1–100 ng ml^{-1}	No
Dexametasone	10^{-10} to 10^{-5} M	No
17-β estradiol	0.5–500 ng ml^{-1}	No
Cytokines/growth factors		
hr IL 1-α	0.01–10 ng ml^{-1}	No
hr IL 1-β	0.01–10 ng ml^{-1}	No
hr IL 2	0.05–5 ng ml^{-1}	No
hr IL 6	0.05–10 ng ml^{-1}	No
hr IL 10	1–30 ng ml^{-1}	No
hr TNF-α	0.01–5 ng ml^{-1}	No
Platelet lysates		
Bovine	100 μg ml^{-1}	Yes
Human	400 μg ml^{-1}	Yes
Other		
Phorbol-myristate-acetate	1–1000 nM	No
Prostaglandin E_2	10^{-10} to 10^{-5} M	No

Table 2
Activity of several platelet factors on the synthesis of BLVp24 in NBC-10 cells.

Platelet factor	Dose range	Stimulatory activity
hr PDGF	1–500 ng ml^{-1}	No
Platelet activating factor	0.05–50 μg ml^{-1}	No
Platelet factor 4	5–5000 ng ml^{-1}	No
hr PD-ECGF	10–1000 ng ml^{-1}	No
Thrombospondin	50–2500 ng ml^{-1}	No
β-Thromboglobulin	5–5000 ng ml^{-1}	No
hr TGF-β_1	>0.5 ng ml^{-1}	Yes
hr TGF-β_2	>0.5 ng ml^{-1}	Yes
hr TGF-α	0.05–50 ng ml^{-1}	No
hr IGF-II	1–500 ng ml^{-1}	No
hr RANTES	12–1500 ng ml^{-1}	No

Each of the substances was tested at various concentrations both in cultures of NBC-10 cells not supplemented with FCS, and also in cultures supplemented with 10% of FCS from a batch without stimulatory activity.

Plasma was obtained from three BLV-free cows after centrifugation (2000 × *g* for 15 min at 4 °C) of heparanized blood, stored at −20 °C and heated at 60 °C for 30 min before used. Preliminary experiments using PBMC as indicator cells showed that the blocking activity of bovine plasma was increased significantly by heating the samples at 60 °C for 30 min. Blood serum was obtained from four BLV-free cows and stored at −20 °C.

Milk obtained from two BLV-free Holstein cows was centrifuged (1000 × *g*, 20 min at 4 °C). The resulting supernatant fat and the sediment were discarded and the samples were maintained at −20 °C until used. Whey was obtained after ultracentrifugation (100,000 × *g*, 90 min at 4 °C) of the milk samples and stored at −20 °C.

The following proteins purified from bovine milk were purchased from Sigma and were tested for inhibitory activity on the synthesis of BLVp24 in the range of concentration indicated between parentheses: lactoferrin (1–500 μg ml^{-1}), α-lactalbumin (0.02–1.5 mg ml^{-1}), β-lactoglobulin (0.2–3 mg ml^{-1}), α-casein (0.1–5 mg ml^{-1}), β-casein (0.2–3 mg ml^{-1}) and κ-casein (0.2–3 mg ml^{-1}). Alpha-1 acid glycoprotein purified from bovine serum was also obtained from Sigma and was tested at 0.1–1000 μg ml^{-1}. The ranges of concentration given comprise the concentration at which each protein is present in 30% milk. The concentration of the main proteins of bovine milk is reported in Swaisgood (1995). The purity of the proteins tested was over 90%.

2.11. Preparation of platelet lysates

Blood was obtained by jugular venipuncture using sodium citrate/EDTA as anticoagulant from a cow that was negative for BLV as determined by the ELISA 108. The platelet-enriched plasma, obtained after the blood was centrifuged at 400 × *g* at 10 °C for 9 min, was harvested and centrifuged at 3000 × *g* for 20 min. The pellet (platelet-rich fraction) was washed twice with PBS and suspended in PBS. Platelets were lysed by five cycles of freezing and thawing. The platelet extract was clarified by centrifugation (12,000 × *g*, 5 min) and protein concentration was determined as described for the cell extracts.

2.12. Neutralization of the stimulatory activity of FCS and PLy with specific antibodies

Neutralization experiments were carried using anti-human TGF-β_1 antibodies developed in chicken and purified by TGF-β_1 affinity chromatography (Sigma). Antibodies were pre-incubated at appropriate dilutions with FCS, PLy or recombinant TGF-β_1 for 15 min at room temperature. NBC-10 cells were then added to the pre-incubated mixtures, and cultures were incubated for 24 h. The final concentration of anti-TGF-β_1 antibodies in the culture medium was 500 ng ml^{-1}. Normal chicken serum was used as control.

2.13. Statistical analysis

Analysis of variance (ANOVA) was used to establish statistical significance for the comparison of the effect of three or more treatments. When interaction between two sources of variation was significant, the main effect was studied under a hierarchical model. Comparisons between pairs of treatments were done by *t*-test. *p* values were two-tailed and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Characterization of cell line NBC-10

Amplification with primer pairs VHF/FR3 rev and IR 0.5 for/IR 1 rev gave consistently products of 341 and 582 bp, respectively in all the samples tested, indicating the presence of genomic DNA. Amplification of genomic DNA obtained from NBC-10 cells with primers VHF and IR1 rev gave consistently a product of about 1 kb, indicating that the V, D and J genes of the IgH locus are rearranged in this cell line (Fig. 1a). Furthermore, flow cytometric analysis with monoclonal antibodies showed that about 30% of these cells express IgM in their membrane (Fig. 1b and c).

3.2. Culture conditions affecting the synthesis of BLVp24 in NBC-10 cells

Previous studies (Ferrer et al., 1971, 1973) have shown that the culture conditions influence the synthesis of BLV particles and BLV antigens in the NBC-10 cell line. Thus, the first objective of the present study was to identify and standardize conditions that stimulate and inhibit the synthesis of BLVp24 in this cell line.

We observed that, when grown as stationary cultures in the presence of certain commercial batches of FCS, such as batch nos. 0051, 0181, 9721, 9722, 9761 and 9941, the NBC-10 cells did not synthesize detectable levels of BLVp24 or synthesized very low levels of this protein (less than 32 ng mg^{-1} of total protein). However, when other commercial batches of FCS, such as batch nos. 9861 and 9862, were used to supplement the culture medium, the NBC-10 cells consistently synthesized high levels of BLVp24 (usually more than 1000 ng mg^{-1} of total protein). According to their activity on the expression of BLVp24 in

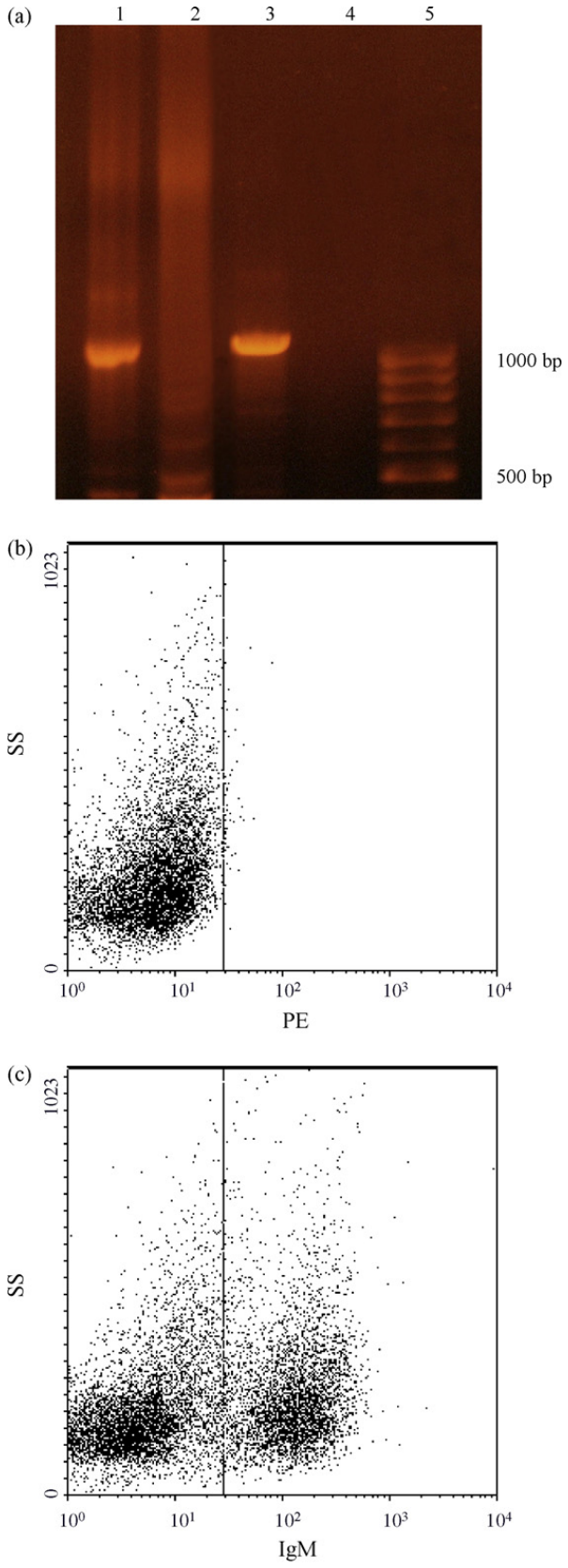

Fig. 1. Molecular and phenotypic characterization of NBC-10 cells. (a) Gel electrophoresis of PCR amplification of the variable region IgH locus. DNA extracted from bovine spleen (positive control, lane 1), MDBK cells (negative control, lane 2) and NBC-10 cells (lane 3) was amplified with primers VHF and IR1 rev as described in Section 2. Lane 4 is a negative control in which DNA was omitted from the amplification reaction. Lane 5 is a marker DNA. (b) Dot plot displayed as side scatter (SS) vs. intensity of PE fluorescence showing the binding of PE-conjugated anti-mouse IgG secondary antibody to NBC-10 cells. Only 1.23% of the cells were stained with the PE-secondary antibody. (c) Dot plot of surface-stained NBC-10 cells displayed as side scatter (SS) vs. intensity of IgM staining. 30.2% of the cells were positive for membrane IgM.

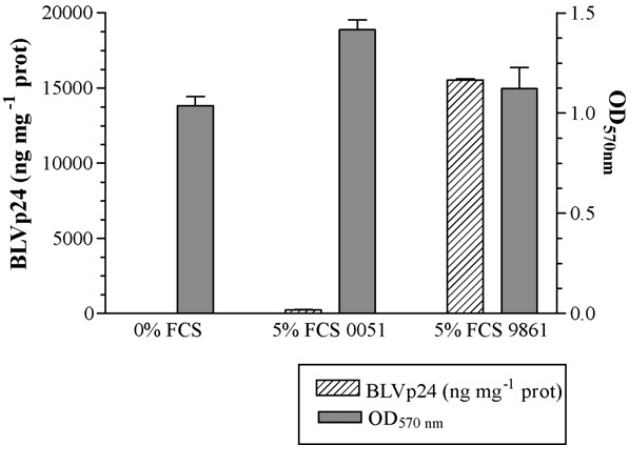

Fig. 2. Synthesis of BLV p24 and number of metabolically active cells ($OD_{570\ nm}$) in 24 h cultures of NBC-10 cells supplemented with different batches of FCS. NBC-10 cells were cultured in the absence of FCS or in the presence of FCS from batches with (batch no. 9861) or without (batch no. 0051) stimulatory activity on the synthesis of BLVp24. BLVp24 was quantified in cell extracts applying the capture ELISA (dashed bars). The reduction of MTT ($OD_{570\ nm}$, solid bars) was assessed in parallel cultures. Bars represent the mean ± S.D. of duplicate (BLVp24 concentration) or triplicate ($OD_{570\ nm}$) cultures.

NBC-10 cells, the batches of FCS were designated as stimulatory and non-stimulatory. The level of BLVp24 synthesized by the NBC-10 cells grown in the presence of stimulatory FCS increased with the concentration of this serum, reaching consistently a maximum at a 10% concentration (data not shown).

To discern if the different levels of BLVp24 detected in NBC-10 cultures supplemented with different batches of FCS reflected changes in the number of viable cells in the cultures with the ability to synthesize the viral protein, the degree of cellular proliferation was determined in parallel cultures using the MTT assay. Fig. 2 shows results from a representative experiment. Regardless of whether or not the FCS used had stimulatory activity on the synthesis of BLVp24, the number of metabolically active cells did not differ significantly between cultures, as determined by the reduction of MTT. These cultures did not show either significant differences in cell concentration or viability (data not shown). However, in parallel cultures, BLVp24 was only detected in high levels in the cultures supplemented with the batch of FCS with stimulatory activity.

NBC-10 cells grown in the presence of non-stimulatory FCS were then used as the indicator system in an assay to detect factors that influence the synthesis of BLVp24. The assay was conducted as described in Section 2. Cultures of NBC-10 cells obtained from stock cultures grown in the presence of non-stimulatory FCS were set in duplicate wells for each variable. Cultures of NBC-10 cells without FCS and/or supplemented with 10% of non-stimulatory FCS were set as negative control. As positive control, NBC-10 cultures were supplemented with 10% of stimulatory FCS

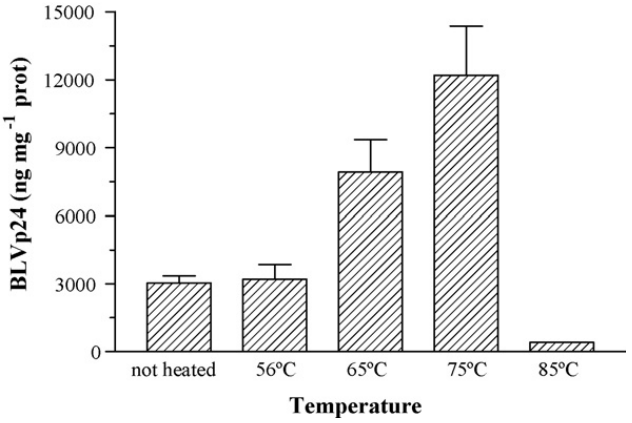

Fig. 3. Effect of the heating temperature on the stimulatory activity of FCS. Aliquots of FCS from batch no. 9861 (with stimulatory activity) were incubated for 30 min at the indicated temperatures. BLVp24 concentration was determined by the capture ELISA in cell extracts prepared from 24 h cultures of NBC-10 cells supplemented with 10% of the heat-treated aliquots of FCS. Each bar represents the mean ± S.D. of duplicate cultures.

(usually from batch no. 9861). The synthesis of BLVp24, as determined by the capture ELISA, was used as indicator of the degree of expression of the viral genome.

3.3. Characterization of the stimulatory activity of FCS on the synthesis of BLVp24 in NBC-10 cells

To explain the differences in the stimulatory activity of different batches of FCS on the synthesis of BLVp24 in the NBC-10 cells, we considered the possibility that the factor(s) responsible for this activity is partially inactivated under certain conditions. To test this possibility, we studied the effect of temperature and time of storage of the FCS on its ability to stimulate the synthesis of BLVp24 in the NBC-10 cells.

Aliquots of FCS from a batch with strong stimulatory activity were heated at different temperatures for 30 min and then used to supplement the culture medium of 24 h cultures of NBC-10 cells. Fig. 3 shows results from a representative experiment. The stimulatory activity of FCS increased significantly as the heating temperature increased, with the greatest activity obtained when this temperature was 75 °C. However, the activity was drastically reduced when the FCS was heated at 85 °C.

Other experiments (not shown) showed that the stimulatory activity was completely lost when the FCS was stored at 37 °C for at least 18 h before use. However, the FCS retained its stimulatory activity when was heated at 70 °C for 30 min before being stored at 37 °C for 18 h. Heating at temperatures below 70 °C did not prevent the loss of activity that occurred when the FCS was stored at 37 °C. On the basis of these observations, to preserve their stimulatory activity, the batches of FCS were stored at −20 °C and heated at 70 °C for 30 min before use.

3.4. Stimulatory activity in sera from fetuses obtained at slaughterhouse

The presence of stimulatory activity on the synthesis of BLVp24 in NBC-10 cultures was examined in serum samples obtained at slaughterhouse from 15 fetuses of various gestational ages. When examined at a 10% concentration, stimulatory activity was demonstrated in all these samples, but it was significantly higher in samples from fetuses in the second trimester of gestation than in those from 6- to 9-month-old fetuses ($p < 0.05$).

3.5. Stimulatory activity of various substances on the synthesis of BLVp24 in NBC-10 cells

Table 1 shows the activity of various substances on the expression of BLVp24 in NBC-10 cells. These substances included those that in previous studies (Stock and Ferrer, 1972; Chatterjee et al., 1985; Tsukiyama et al., 1987; Lagarias and Radke, 1989; Kidd and Radke, 1996; Pyeon et al., 2000) have shown to induce the expression of BLV in cultures of PBMC (mitogens, platelet lysates, prostaglandin E_2 and phorbol esters). Hormones, cytokines and growth factors, some of which have also been shown to influence the *in vitro* expression of BLV (Meirom et al., 1997; Niermann and Buehring, 1997; Trueblood et al., 1998; Pyeon and Splitter, 1999; Pyeon et al., 2000) were also included. All the substances were tested in a wide range of concentrations. Only PLy showed stimulatory activity. Bovine PLy stimulated the synthesis of BLVp24 at the same level than FCS from batch no. 9861 did ($p < 0.05$).

In order to identify the factor(s) responsible for the stimulatory activity of PLy on the synthesis of BLVp24 in the NBC-10 cells, several commercially available platelet factors were tested. Most of these factors were produced by genetic recombination or were highly purified from human platelets. As shown by the results summarized in Table 2, of the platelet factors tested, only TGF-β_1 and TGF-β_2 showed stimulatory activity. The other mammalian isoform of TGF-β (TGF-β_3) also stimulated the synthesis of BLVp24 in NBC-10 cells. There were no significant differences between the stimulatory activities of the three isoforms of TGF-β ($p < 0.05$). Other members of the TGF-β superfamily, such as activin and inhibin, tested at a wide concentration range, did not show stimulatory activity.

3.6. Neutralization of the stimulatory activity of FCS and PLy by anti-TGF-β antibodies

Neutralization experiments were conducted to determine if TGF-β was the factor responsible for the stimulatory activity of FCS and PLy on the synthesis of BLVp24 in NBC-10 cells. These experiments were carried out using commercial polyclonal antibodies prepared by immunizing chicken with recombinant TGF-β and purified by affinity chromatography.

As shown in Table 3, the synthesis of BLVp24 induced by either FCS (batch no. 9861), PLy or recombinant TGF-β was strongly neutralized by anti-TGF-β antibodies, but not by normal chicken sera.

3.7. Stimulatory activity of TGF-β on the synthesis of BLVp24 in short-term PBMC cultures

Previous studies have shown that the expression of BLVp24 in cultures of PBMC from BLV-infected cows is

Table 3
Neutralization of the synthesis of BLVp24 in NBC-10 cells by chicken serum with antibodies against the transforming growth factor-β_1 (TGF-β_1).

BLVp24 synthesis induced by	Concentration	Serum tested for neutralizing activity	BLVp24 (ng mg^{-1} total prot)[a]	% Control[b]
FCS #9861	7.5%	None	963.5 ± 11.5	100
FCS #9861	7.5%	Normal chicken serum	848.5 ± 147.5	88.1
FCS #9861	7.5%	Serum anti-TGF-β_1	168.5 ± 28.5	17.5
Platelet lysate	325 μg ml^{-1}	None	2259 ± 232	100
Platelet lysate	325 μg ml^{-1}	Normal chicken serum	2660.5 ± 21.5	117.8
Platelet lysate	325 μg ml^{-1}	Serum anti-TGF-β_1	231 ± 24	10.2
rTGF-β_1	2 ng ml^{-1}	None	476 ± 22	100
rTGF-β_1	2 ng ml^{-1}	Normal chicken serum	544 ± 156	114.3
rTGF-β_1	2 ng ml^{-1}	Serum anti-TGF-β_1	61 ± 45	12.8

[a] Mean ± S.D. of duplicate cultures.
[b] Mean of duplicate cultures.

greatly stimulated by the addition of FCS (Zandomeni et al., 1992) or PLy (Tsukiyama et al., 1987) to the culture medium.

To further evaluate the activity of TGF-β on the expression of BLV, recombinant TGF-β was tested in short-term cultures of PBMC obtained from five cows with persistent lymphocytosis. FCS from batch no. 9861 (with stimulatory activity) at 10% concentration was used as positive control. As shown in Table 4, recombinant TGF-β stimulated the expression of BLVp24 in PBMC cultures from four of five donors tested. The level of stimulation observed varied between 1.7- and 10-fold.

3.8. Inhibition of the synthesis of BLVp24 induced by FCS, PLy and recombinant TGF-β in NBC-10 cells by host soluble factors

The inhibitory activity of bovine plasma on the expression of BLV has been previously demonstrated in short-term cultures of PBMC (Gupta and Ferrer, 1982; Zandomeni et al., 1992, 1994; Taylor and Jacobs, 1993). To determine if bovine plasma also inhibits the expression of BLV in NBC-10 cells, cell cultures were established and the synthesis of BLVp24 was stimulated by FCS (batch no. 9861), PLy or recombinant TGF-β. In these experiments, plasma from a BLV-negative cow was added at a final concentration of 30% at the onset of parallel cultures. As shown in Fig. 4a, this plasma strongly inhibited the synthesis of BLVp24 induced by FCS, PLy or recombinant TGF-β. To determine if this inhibition was associated with a decrease in the number of viable cells, the reduction of MTT was measured in parallel cultures. As shown in Fig. 4b, although the synthesis of BLVp24 induced by FCS was almost completely abolished in the cultures supplemented with bovine plasma, the number of metabolically active cells was not reduced in these cultures.

The inhibitory activity on the expression of BLVp24 was also demonstrated on blood serum obtained from four BLV-free cows, although the level of inhibitory activity was significantly lower than that observed in plasma ($p < 0.01$).

As shown in Fig. 5a the synthesis of BLVp24 induced in NBC-10 cells by FCS (batch no. 9861) was strongly inhibited by milk and, to a lesser extent, by whey of BLV-free cows, even when these fluids were added to the cultures at concentrations as low as 5%.

In other experiments we found that whey from BLV-free cattle added at a final concentration of 30% to the culture medium also inhibits the synthesis of BLVp24 induced by PLy. The inhibition by whey was significantly less pronounced when the synthesis of BLVp24 was induced by recombinant TGF-β ($p < 0.05$).

An experiment was conducted to determine if the inhibition of the synthesis of BLVp24 in cultures of NBC-10 cells by whey was due to a toxic effect of this fluid. As shown in Fig. 5b, while the addition of whey almost completely abolished the synthesis of BLVp24 induced by

Table 4
Induction of the synthesis of BLVp24[a] in 24 h cultures of PBMC from BLV-infected cows by recombinant TGF-β .

Donor #	Exp #	Non-stimulated	TGF-β_1				FCS 10%
			2 ng ml^{-1}	4–10 ng ml^{-1}	15 ng ml^{-1}	50 ng ml^{-1}	
1	1	154.5 ± 46	132.5 ± 105	64.5 ± 10	ND	ND	218.5 ± 100
	2	88.5 ± 1,5	185.6 ± 23	231.8 ± 36	ND	ND	562 ± 6
2	1	441 ± 16	969.5 ± 38	ND	816 ± 16	ND	729 ± 17
3	1	495.5 ± 29	ND	4381.5 ± 1559	ND	4126.5 ± 1426	2901 ± 948
	2	694.5 ± 143	2925 ± 796	6763 ± 1075	6489 ± 819	ND	3868.5 ± 1589
	3	922.5 ± 179	ND	6597.5 ± 1248	ND	ND	7994.5 ± 279
4	1	1725.5 ± 45	1936.5 ± 28	ND	1709.5 ± 95	ND	3165 ± 42
5	1	518 ± 88	1830.5 ± 416	ND	2600.5 ± 162.5	ND	3493 ± 876
	2	1212 ± 62	ND	1903 ± 369	ND	2159.5 ± 815	1636.5 ± 107
	3	5925.5 ± 27	ND	7683.5 ± 850	10,296 ± 297	ND	6497.5 ± 953

[a] BLVp24 (ng mg^{-1} total protein) (mean ± S.D. of duplicate cultures).

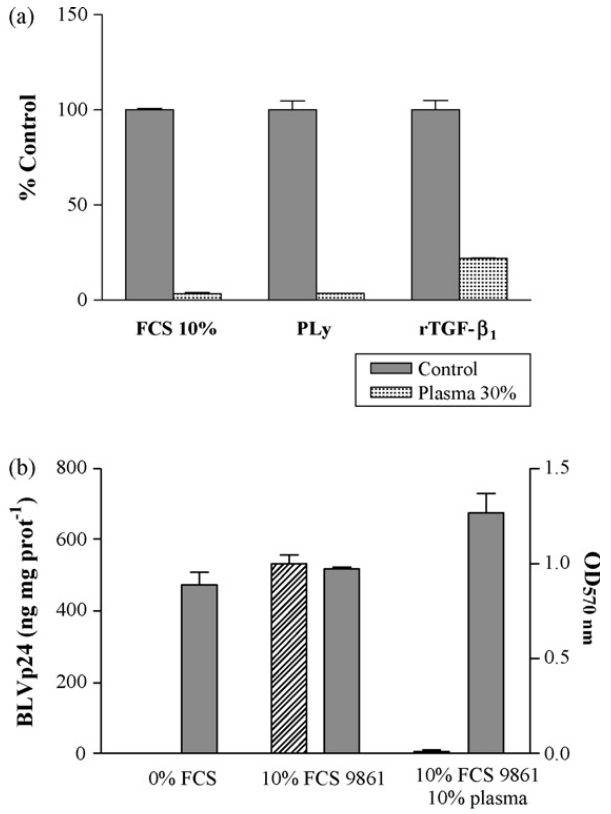

Fig. 4. Effect of plasma from a BLV-negative cow on the synthesis of BLVp24 and number of metabolically active cells in 24 h cultures of NBC-10 cells. (a) The synthesis of BLVp24 was induced by 10% of FCS (batch no. 9861), PLy (324 μg ml^{-1}) or recombinant TGF-β_1 (5 ng ml^{-1}). Bovine plasma (final concentration 30%) was added at the onset of the cultures. Bars represent the mean ± S.D. of duplicate cultures. (b) The synthesis of BLVp24 (dashed bars) was quantified in duplicate cultures supplemented with 10% of FCS (batch no. 9861), 10% FCS plus 10% bovine plasma, or S-MEM alone. The number of metabolically active cells was assessed by measuring the reduction of MTT in triplicate parallel cultures (OD570 nm, solid bars). Bars represent the mean ± S.D.

FCS in cultures of NBC-10 cells, the number of metabolically active cells, as determined by the MTT assay in parallel cultures not treated with whey, was not significantly reduced ($p < 0.05$). In addition, the cell viability in the cultures supplemented with whey was above 90%.

Most of the commercially available main components of bovine milk whey were tested for inhibitory activity on the synthesis of BLVp24 in NBC-10 cells. These components included proteins, such as lactoferrin, α-lactalbumin, β-lactoglobulin and α_1-acid glycoprotein, and hormones such as progesterone and dexametasone. None of these substances showed inhibitory activity at any of the concentrations tested. (The ranges of concentrations tested comprised the concentration at which each protein is present in 30% milk. This percentage of milk results in almost complete inhibition of BLVp24 synthesis.) On the other hand, as shown in Fig. 6a, the synthesis of BLVp24 induced by FCS in NBC-10 cells was inhibited by the β fraction of bovine milk casein in a dose-dependent manner. The same inhibitory effect of β-casein was observed when recombinant TGF-β was used to induce the synthesis of BLVp24 (Fig. 6b). No inhibitory activity was detected in the α and κ fractions of casein when tested in a concentration range equivalent to that in which they are present in milk.

3.9. Discussion

In the present study we confirm previous reports (Ferrer et al., 1971, 1973) showing that BLV expression in NBC-10 cells is highly influenced by the culture conditions. Previously reported studies (Hare et al., 1968) showed that these cells are anueploid and derived from neoplastic cells of the diseased animals. The findings in the present study that the genes encoding for the variable region of the IgH chain are re-arranged in the NBC-10 cells and that a subpopulation of these cells express IgM in their membrane show that they are B-lymphoid cells.

We demonstrate that the addition of FCS from certain batches and PLy stimulate the synthesis of BLVp24 in NBC-10 cultures, and that the increase in the synthesis of BLVp24 resulting from the addition of FCS to these cultures is not associated with changes in the number of metabolically active cells. Thus, it seems reasonable to postulate that this increase is a consequence of the transcriptional or post-transcriptional de-repression of the BLV genome. Further studies are needed to distinguish between these two possibilities.

Our inhibition experiments with specific antibodies demonstrate that TGF-β is mainly responsible for the stimulatory activity of FCS and PLy on the synthesis of BLVp24 in the NBC-10 cells.

Moulin et al. (1997) have detected high levels of latent TGF-β in fetal bovine serum, thus suggesting the possibility that TGF-β is a normal component of this serum. Consistent with this possibility is the observation that the three isoforms of TGF-β are secreted by cells of bovine placentas (Munson et al., 1996). Thus, it seems reasonable to postulate that TGF-β is responsible for the stimulatory activity on the synthesis of BLVp24 in NBC-10 cultures detected in the serum of all the fetuses examined in the present study. The finding that the level of this stimulatory activity is significantly higher in sera from fetuses in the second trimester of gestation than in the third trimester suggests that the synthesis of TGF-β by the placenta decreases as gestation progresses.

It has been shown that TGF-β is secreted in a latent form that it must be activated to produce biologically active TGF-β Such activation can be achieved by several physiologic mechanisms (reviewed in: Gleizes et al., 1997; Murphy-Ullrich and Poczatek, 2000) or by physiochemical treatments, including heat (Brown et al., 1990). Our results showing that the activity of FCS on the expression of BLV is greatly enhanced after heating the FCS at 75 °C are consistent with the possibility that latent TGF-β is activated at this temperature. It is conceivable that the loss of stimulatory activity observed in the FCS stored at 37 °C for more than 18 h is due to the inactivation of a factor (i.e. plasmin, cathepsin, or another enzyme) required for the *in vitro* activation of latent TGF-β. Heating the FCS at 70 °C before its storage at 37 °C would activate the latent TGF-β, which then would exert its biological activity without the need of further activation during the culture.

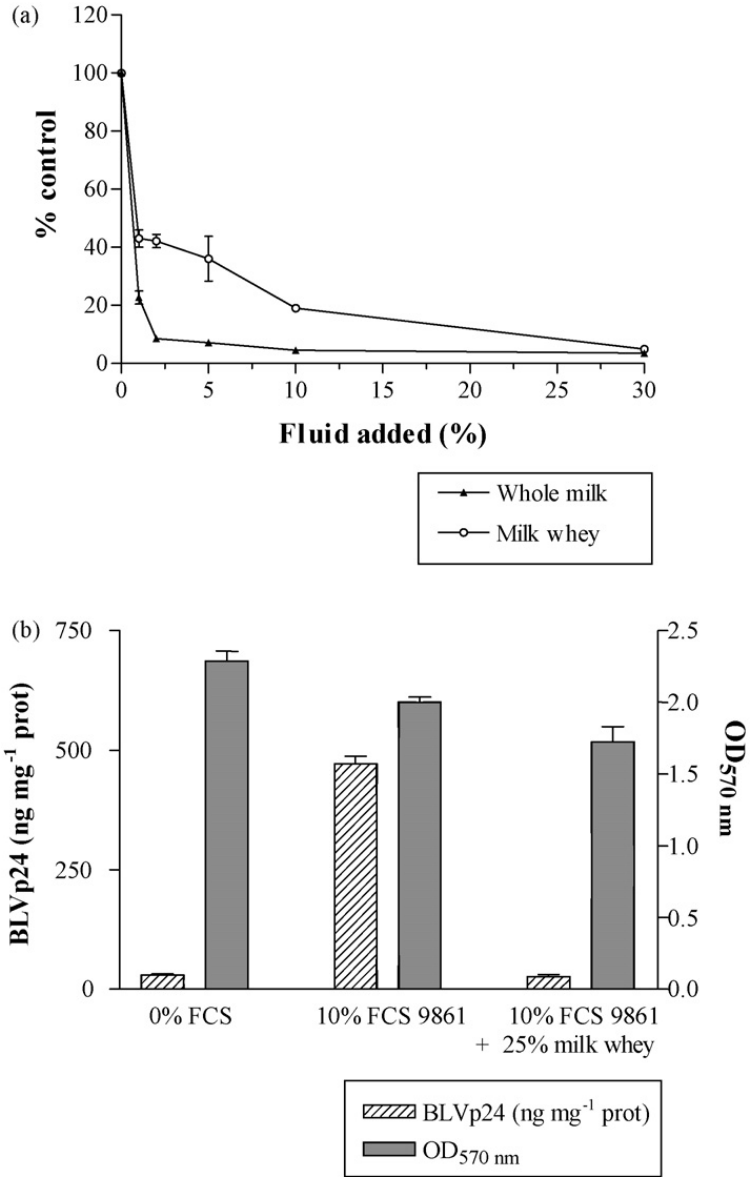

Fig. 5. Effect of bovine milk whey on the synthesis of BLVp24 and number of metabolically active cells in 24 h cultures of NBC-10 cells. (a) Inhibitory activity of bovine milk and whey on the synthesis of BLVp24 in NBC-10 cells. The synthesis of BLVp24 was induced by FCS (batch no. 9861). Whole milk and whey were added at the indicated concentrations at the onset of duplicate cultures. (b) The synthesis of BLVp24 (dashed bars) was quantified in duplicate cultures supplemented with 10% of FCS (batch no. 9861), 10 % FCS and milk whey or S-MEM alone. The number of metabolically active cells was assessed by measuring the reduction of MTT in triplicate parallel cultures ($OD_{570\ nm}$, solid bars). Bars represent the mean ± SD.

As it has been reported previously, FCS (Zandomeni et al., 1992) and platelet extracts (Tsukiyama et al., 1987; Zandomeni et al., 1992) also stimulate the synthesis of BLVp24 in short-term PBMC cultures. In the present study we demonstrate that in most cases the expression of BLV in PBMC cultures can also be induced by the addition of TGF-β. The stimulatory activity of TGF-β on the synthesis of BLVp24 was also demonstrated in another B-cell line naturally infected with BLV, named NBC-13 (data not shown). The characteristics of BLV expression in this cell line have been reported (Ferrer et al., 1971, 1973; Jensen et al., 1992).

TGF-β has been shown to enhance the replication of HTLV-I in human PBMC cultures. This seems to occur, at least in part, as a result of the transactivation of the viral LTR (Moriuchi and Moriuchi, 2002). It has been reported that TGF-β induces the expression of the glucose transporter type 1, the receptor for HTLV-I, on quiescent naïve T cells (Jones et al., 2005). The possibility that similar mechanisms are involved in the activation of BLV expression by TGF-β has not been studied.

Substances such as mitogens, PMA and PGE_2 that in previous studies stimulated the expression of BLV in PBMC cultures from infected cattle (Miller et al., 1969; Stock and Ferrer, 1972; Baliga and Ferrer, 1977; Driscoll et al., 1977; Chatterjee et al., 1985; Djilali et al., 1987; Lagarias and Radke, 1989; Jensen et al., 1990; Kidd and Radke, 1996; Pyeon et al., 2000) did not induce the synthesis of BLVp24 in NBC-10 cultures. It seems that the diversity of factors capable of inducing the synthesis of BLV in PBMC cultures is greater than in the NBC-10 cells. This is probably due to the presence in the PBMC cultures of cell subsets other

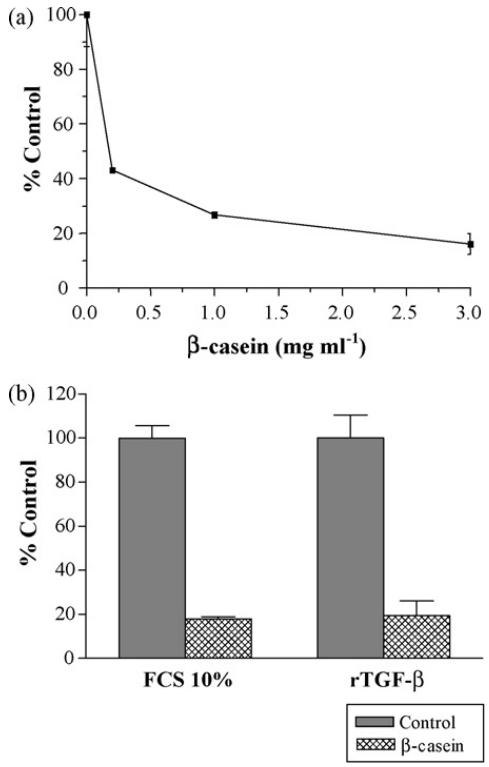

Fig. 6. Effect of purified β-casein on the synthesis of BLVp24 in 24 h cultures of NBC-10 cells. (a) The synthesis of BLVp24 was induced by 10% of FCS (batch no. 9861). β-Casein was added at the indicated concentrations at the onset of cultures. Each dot represent the mean ± S.D. of duplicate cultures. (b) The synthesis of BLVp24 was induced by 10% of FCS (batch no. 9861) or recombinant TGF-β_1 (2 ng ml^{-1}). β-Casein was added at a final concentration of 3 mg ml^{-1} at the onset of cultures. Bars represent the mean ± S.D. of duplicate cultures.

than B cells. Indeed, TGF-β is secreted by T cells, B cells and monocytes activated by several stimuli, including PHA, LPS, ConA, anti-IgM antibodies and phorbol esters (Kehrl et al., 1986; Lucas et al., 1990; Sing et al., 1990; Letterio and Roberts, 1998). Thus, TGF-β secreted in the PBMC cultures upon activation with any of these factors could be, at least in part, responsible for the de-repression of BLV. The finding in the present study that exogenous TGF-β also stimulates the synthesis of BLVp24 in cultures of PBMC is consistent with this possibility.

It is possible that, as the result of the stimulation of the synthesis of TGF-β by physiological or external factors, BLV expression occasionally occurs in the host. It remains to be determined if this stimulation plays a role in the biology and pathology of BLV.

A potential mechanism of HTLV-I induced leukemogenesis has been proposed involving HTLV-I Tax and TGF-β. HTLV-I Tax has been shown to transactivate the TGF-β promoter (Kim et al., 1990) resulting in an increased production of TGF-β in cells from HTLV-I-induced adult T-cell leukemia patients as well as HTLV-I infected cell lines (Niitsu et al., 1988; Kim et al., 1990). In contrast to normal peripheral T cells, HTLV-I infected cells are resistant to the growth inhibitory effects of TGF-β, as HTLV-I Tax represses TGF-β signaling (Hollsberg et al., 1994; Mori et al., 2001; Arnulf et al., 2002). The consequent high proliferation state of HTLV-I infected cells could be the initial step in HTLV-I induced leukemogenesis, allowing the clonal expansion of infected cells and the subsequent accumulation of oncogenic events (Arnulf et al., 2002). Although caution should be exercised in extrapolating information from a system, such as is the HTLV system, in which the target cells are T cells, to the BLV system which involves B target cells, the possibility should be considered that TGF-β may also play a role in BLV-induced leukemogenesis through a mechanism involving BLV Tax.

We demonstrate that, as it was previously found in short-term cultures of PBMC from BLV infected cattle (Gupta and Ferrer, 1982; Gupta et al., 1984; Zandomeni et al., 1992, 1994), plasma and serum from BLV-negative cattle block the synthesis of BLVp24 induced by FCS in the NBC-10 cells. Bovine plasma also blocks the synthesis of BLVp24 induced by PLy or recombinant TGF-β in these cells. van den Heuvel et al. (2005) have shown that a fibronectin-containing complex isolated from bovine plasma blocks the synthesis of BLVp24 in short-term cultures of bovine PBMC. It remains to be determined if this complex also has blocking activity on the synthesis of BLVp24 in cultures of NBC-10 cells.

In the present study we have found that bovine milk, milk whey and the β fraction of casein have a potent blocking activity on the synthesis of BLVp24 in NBC-10 cell cultures. The fact that peptides derived from β-casein are present in the proteose-peptone fraction of bovine whey (Andrews, 1978a,b; Eigel and Keenan, 1979) is consistent with the idea that the blocking activity of this fluid may be due, at least in part, to these peptides.

It was not possible to determine if the whey and β-casein also block the synthesis of BLVp24 in PMBC cultures because both have a strong stimulatory activity in these cultures (data not shown). Further studies are needed to determine if the stimulatory activity of whey and β-casein on the expression of BLV in the PBMC cultures is due to the presence of TGF-β in milk (Rogers et al., 1996), or to the presence in this fluid of other factors lacking stimulatory activity in the NBC-10 cultures. Further studies are also needed to determine the relationship of the factor(s) responsible for the blocking activity of bovine whey and β-casein on the synthesis of BLVp24 in NBC-10 cells to the factor(s) (PBB) found in plasma from cattle and human and in bovine lymphatic fluid with blocking activity on the expression of BLV in short-term PMBC cultures (Gupta and Ferrer, 1982; Gupta et al., 1984; Zandomeni et al., 1992, 1994). While it is conceivable that different factors are responsible for the BLV blocking activity of plasma, whey and lymphatic fluid, the possibility should be considered that the BLV blocking factor is a specific peptide or a polypeptide shared by β-casein with proteins present in these fluids and in the plasma fibronectin-containing complex described by van den Heuvel et al. (2005). If this possibility is confirmed, the observation that the α and κ fractions of casein lack blocking activity would provide an important lead for the precise identification and characterization of the BLV blocking factor(s).

On the basis of the data reported here and in previous studies (Gupta and Ferrer, 1982; Gupta et al., 1984; Zandomeni et al., 1992, 1994), it seems clear that the BLV blocking factor(s) exert its activity mainly by interfering

with the activity of factors, such as TGF-β, that stimulate the expression of the virus.

The data obtained in the present study also shows that the NBC-10 cells provide a reliable indicator system for a reproducible and practical assay to detect factors with blocking and stimulating activity on the synthesis of BLVp24 in naturally infected B cells.

Since the factors that block the expression of BLV *in vitro* are present in body fluids, such as plasma, serum and milk from BLV-free cattle (Gupta and Ferrer, 1982; Gupta et al., 1989; Zandomeni et al., 1992; Taylor and Jacobs, 1993; and this paper), it is evident that these factors are not induced by the viral infection. It is therefore reasonable to assume that the host soluble blocking factors have evolved as a natural mechanism of resistance to the expression and replication of the virus and, therefore, to the induction of the disease(s) it causes. Indeed, it is conceivable that the lesser the virus expression and replication in the host, the lower the probability that the virus may infect cells susceptible to neoplastic transformation. At the same time, the repressed state of the virus induced by the blocking factors *in vivo* may be the mechanism by which virus infection becomes persistent in the face of a host immune response.

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References

Abbott, M.A., Poiesz, B.J., Byrne, B.C., Kwok, S., Sninsky, J.J., Ehrlich, G.D., 1988. Enzymatic gene amplification: qualitative and quantitative methods for detecting proviral DNA amplified in vitro. J. Infect. Dis. 158, 1158–1169.

Andrews, A.T., 1978a. The composition, structure and origin of proteose-peptone component 5 of bovine milk. Eur. J. Biochem. 90, 59–65.

Andrews, A.T., 1978b. The composition, structure and origin of proteose-peptone component 8F of bovine milk. Eur. J. Biochem. 90, 67–81.

Arnulf, B., Villemain, A., Nicot, C., Mordelet, E., Charneau, P., Kersual, J., Zermati, Y., Mauviel, A., Bazarbachi, A., Hermine, O., 2002. Human T-cell lymphotropic virus oncoprotein Tax represses TGF-beta 1 signaling in human T cells via c-Jun activation: a potential mechanism of HTLV-I leukemogenesis. Blood 100, 4129–4138.

Baliga, V., Ferrer, J.F., 1977. Expression of the bovine leukemia virus and its internal antigen in blood lymphocytes. Proc. Soc. Exp. Biol. Med. 156, 388–391.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brown, P.D., Wakefield, L.M., Levinson, A.D., Sporn, M.B., 1990. Physicochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and, 3. Growth Factors 3, 35–43.

Chatterjee, R., Gupta, P., Kashmiri, S.V., Ferrer, J.F., 1985. Phytohemagglutinin activation of the transcription of the bovine leukemia virus genome requires de novo protein synthesis. J. Virol. 54, 860–863.

Djilali, S., Parodi, A.L., Levy, D., 1987. Bovine leukemia virus replicates in sheep B lymphocytes under a T cell released factor. Eur. J. Cancer Clin. Oncol. 23, 81–85.

Driscoll, D.M., Baumgartener, L.E., Olson, C., 1977. Concanavalin A and the production of bovine leukemia virus antigen in short-term lymphocyte cultures. J. Natl. Cancer Inst. 58, 1513–1514.

Eigel, W.N., Keenan, T.W., 1979. Identification of proteose-peptone component 8-slow as a plasmin derived fragment of bovine β-casein. Int. J. Biochem. 10, 529–535.

Ferrer, J.F., 1980. Bovine lymphosarcoma. Adv. Vet. Sci. Comp. Med. 24, 1–68.

Ferrer, J.F., Avila, L., Stock, N.D., 1973. Recent electron microscopic and immunologic studies on bovine cell cultures containing C-type viruses. Bibl. Haematol. 39, 206–214.

Ferrer, J.F., Marshak, R.R., Abt, D.A., Kenyon, S.J., 1979. Relationship between lymphosarcoma and persistent lymphocytosis in cattle: a review. J. Am. Vet. Med. Assoc. 175, 705–708.

Ferrer, J.F., Stock, N.D., Lin, P., 1971. Detection of replicating C-type viruses in continuous cell cultures established from cows with leukemia: effect of the culture medium. J. Natl. Cancer Inst. 47, 613–621.

Ghezzi, P.C., Dolcini, G.L., Gutierrez, S.E., Bani, P.C., Torres, J.O., Arroyo, G.H., Esteban, E.N., 1997. Bovine leukemia virus (BLV): prevalence in the Cuenca Lechera Mar y Sierras from 1994 to 1995. Rev. Argent. Microbiol. 29, 137–146.

Gleizes, P.E., Munger, J.S., Nunes, I., Harpel, J.G., Mazzieri, R., Noguera, I., Rifkin, D.B., 1997. TGF-beta latency: biological significance and mechanisms of activation. Stem Cells 15, 190–197.

Gupta, P., Chatterjee, R., Cai, Q., 1989. Prevalence of the plasma bovine leukaemia virus blocking factor in cattle from a commercial dairy herd. Vet. Rec. 125, 5–6.

Gupta, P., Ferrer, J.F., 1982. Expression of bovine leukemia virus genome is blocked by a nonimmunoglobulin protein in plasma from infected cattle. Science 215, 405–407.

Gupta, P., Kashmiri, S.V., Ferrer, J.F., 1984. Transcriptional control of the bovine leukemia virus genome: role and characterization of a non-immunoglobulin plasma protein from bovine leukemia virus-infected cattle. J. Virol. 50, 267–270.

Gutierrez, S.E., Dolcini, G.L., Arroyo, G.H., Rodriguez Dubra, C., Ferrer, J.F., Esteban, E.N., 2001. Development and evaluation of a highly sensitive and specific blocking enzyme-linked immunosorbent assay and polymerase chain reaction assay for diagnosis of bovine leukemia virus infection in cattle. Am. J. Vet. Res. 62, 1571–1577.

Hare, W.C., Lin, P.S., Zachariasewycz, E., 1968. Studies on 12 cell lines established from mononuclear leukocytes of cattle with lymphosarcoma. Cancer 22, 1074–1084.

Hollsberg, P., Ausubel, L.J., Hafler, D.A., 1994. Human T cell lymphotropic virus type I-induced T cell activation resistance to TGF-beta 1-induced suppression. J. Immunol. 153, 566–573.

Holy, L., 1983. Capítulo 13: Gravidez, preñez o gestación. Bases Biológicas de la Reproducción. Editorial Diana, México, p. 210.

Jensen, W.A., Sheehy, S.E., Fox, M.H., Davis, W.C., Cockerell, G.L., 1990. In vitro expression of bovine leukemia virus in isolated B-lymphocytes of cattle and sheep. Vet. Immunol. Immunopathol. 26, 333–342.

Jensen, W.A., Wicks-Beard, B.J., Cockerell, G.L., 1992. Inhibition of protein kinase C results in decreased expression of bovine leukemia virus. J. Virol. 66, 4427–4433.

Jones, K.S., Akel, S., Petrow-Sadowski, C., Huang, Y., Bertolette, D.C., Ruscetti, F.W., 2005. Induction of human T cell leukemia virus type I receptors on quiescent naive T lymphocytes by TGF-beta. J. Immunol. 174, 4262–4270.

Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M.B., Fauci, A.S., 1986. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. 163, 1037–1050.

Kettmann, R., Deschamps, J., Cleuter, Y., Couez, D., Burny, A., Marbaix, G., 1982. Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3′

proximate cellular sequences. Proc. Natl. Acad. Sci. U.S.A. 79, 2465–2469.
Kettmann, R., Marbaix, G., Cleuter, Y., Portetelle, D., Mammerickx, M., Burny, A., 1980. Genomic integration of bovine leukemia provirus and lack of viral RNA expression in the target cells of cattle with different responses to BLV infection. Leuk. Res. 4, 509–519.
Kidd, L.C., Radke, K., 1996. Lymphocyte activators elicit bovine leukemia virus expression differently as asymptomatic infection progresses. Virology 217, 167–177.
Kim, S.J., Kehrl, J.H., Burton, J., Tendler, C.L., Jeang, K.T., Danielpour, D., Thevenin, C., Kim, K.Y., Sporn, M.B., Roberts, A.B., 1990. Transactivation of the transforming growth factor beta 1 (TGF-beta 1) gene by human T lymphotropic virus type 1 tax: a potential mechanism for the increased production of TGF-beta 1 in adult T cell leukemia. J. Exp. Med. 172, 121–129.
Lagarias, D.M., Radke, K., 1989. Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections. J. Virol. 63, 2099–2107.
Letterio, J.J., Roberts, A.B., 1998. Regulation of immune responses by TGF-beta. Annu. Rev. Immunol. 16, 137–161.
Lucas, C., Bald, L.N., Fendly, B.M., Mora-Worms, M., Figari, I.S., Patzer, E.J., Palladino, M.A., 1990. The autocrine production of transforming growth factor-beta 1 during lymphocyte activation. A study with a monoclonal antibody-based ELISA. J. Immunol. 145, 1415–1422.
Marshak, R.R., Hare, W.C., Abt, D.A., Croshaw Jr., J.E., Switzer, J.W., Ipsen, I., Dutcher, R.M., Martin, J.E., 1963. Occurrence of lymphocytosis in dairy cattle herds with high incidence of lymphosarcoma. Ann. NY Acad. Sci. 108, 1284–1301.
McDonald, H.C., Ferrer, J.F., 1976. Detection, quantitation and characterization of the major internal virion antigen of the bovine leukemia virus by radioimmunoassay. J. Natl. Cancer Inst. 57, 875–882.
McGahon, A., Martin, S., Bissonnette, R., Mahboubi, A., Shi, Y., Mogil, R., Nishioka, W., Green, D., 1995. The end of the (cell) line: methods for the study of apoptosis in vitro. Meth. Cell Biol. 46, 153–185.
Meirom, R., Moss, S., Brenner, J., Heller, D., Trainin, Z., 1997. Levels and role of cytokines in bovine leukemia virus (BLV) infection. Leukemia 11 (3), 219–220.
Miller, J.M., Miller, L.D., Olson, C., Gillette, K.G., 1969. Virus-like particles in phytohemagglutinin-stimulated lymphocyte cultures with reference to bovine lymphosarcoma. J. Natl. Cancer Inst. 43, 1297–1305.
Mori, N., Morishita, M., Tsukazaki, T., Giam, C.Z., Kumatori, A., Tanaka, Y., Yamamoto, N., 2001. Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300. Blood 97, 2137–2144.
Moriuchi, M., Moriuchi, H., 2002. Transforming growth factor-beta enhances human T-cell leukemia virus type I infection. J. Med. Virol. 67, 427–430.
Moulin, V., Auger, F.A., O'Connor-McCourt, M., Germain, L., 1997. Fetal and postnatal sera differentially modulate human dermal fibroblast phenotypic and functional features in vitro. J. Cell Physiol. 171, 1–10.
Munson, L., Wilhite, A., Boltz, V.F., Wilkinson, J.E., 1996. Transforming growth factor beta in bovine placentas. Biol. Reprod. 55, 748–755.
Murphy-Ullrich, J.E., Poczatek, M., 2000. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. Cytokine Growth Factor Rev. 11, 59–69.
NAHMS, 1996. National Animal Health Monitoring System: high prevalence of BLV in US dairy herds, Available at: http://www.aphis.usda.gov/vs/ceah/cahm/Dairy_Cattle/d96blv.htm.
Niermann, G.L., Buehring, G.C., 1997. Hormone regulation of bovine leukemia virus via the long terminal repeat. Virology 239, 249–258.
Niitsu, Y., Urushizaki, Y., Koshida, Y., Terui, K., Mahara, K., Kohgo, Y., Urushizaki, I., 1988. Expression of TGF-beta gene in adult T cell leukemia. Blood 71, 263–266.
Pyeon, D., Diaz, F.J., Splitter, G.A., 2000. Prostaglandin E(2) increases bovine leukemia virus tax and pol mRNA levels via cyclooxygenase 2: regulation by interleukin-2, interleukin-10, and bovine leukemia virus. J. Virol. 74, 5740–5745.
Pyeon, D., Splitter, G.A., 1999. Regulation of bovine leukemia virus tax and pol mRNA levels by interleukin-2 and -10. J. Virol. 73, 8427–8434.
Rogers, M.L., Goddard, C., Regester, G.O., Ballard, F.J., Belford, D.A., 1996. Transforming growth factor beta in bovine milk: concentration, stability and molecular mass forms. J. Endocrinol. 151, 77–86.
Sing, G.K., Ruscetti, F.W., Beckwith, M., Keller, J.R., Ellingsworth, L., Urba, W.J., Longo, D.L., 1990. Growth inhibition of a human lymphoma cell line: induction of a transforming growth factor-beta-mediated autocrine negative loop by phorbol myristate acetate. Cell Growth Differ. 1, 549–557.
Stock, N.D., Ferrer, J.F., 1972. Replicating C-type virus in phytohemagglutinin-treated buffy-coat cultures of bovine origin. J. Natl. Cancer Inst. 48, 985–996.
Swaisgood, H., 1995. Chapter 5: Nitrogenous components of milk. F. Protein and amino acid composition of bovine milk. Handbook of Milk Composition, pp. 464–472.
Taylor, J.A., Jacobs, R.M., 1993. Effects of plasma and serum on the in vitro expression of bovine leukemia virus. Lab. Invest. 69, 340–346.
Trono, K.G., Perez-Filgueira, D.M., Duffy, S., Borca, M.V., Carrillo, C., 2001. Seroprevalence of bovine leukemia virus in dairy cattle in Argentina: comparison of sensitivity and specificity of different detection methods. Vet. Microbiol. 83, 235–248.
Trueblood, E.S., Brown, W.C., Palmer, G.H., Davis, W.C., Stone, D.M., McElwain, T.F., 1998. B-lymphocyte proliferation during bovine leukemia virus-induced persistent lymphocytosis is enhanced by T-lymphocyte-derived interleukin-2. J. Virol. 72, 3169–3177.
Tsukiyama, K., Onuma, M., Izawa, H., 1987. Effect of platelet-derived factor on expression of bovine leukemia virus genome. Arch. Virol. 96, 89–96.
Van den Broeke, A., Cleuter, Y., Chen, G., Portetelle, D., Mammerickx, M., Zagury, D., Fouchard, M., Coulombel, L., Kettmann, R., Burny, A., 1988. Even transcriptionally competent proviruses are silent in bovine leukemia virus-induced sheep tumor cells. Proc. Natl. Acad. Sci. U.S.A. 85, 9263–9267.
van den Heuvel, M.J., Jefferson, B.J., Jacobs, R.M., 2005. Isolation of a bovine plasma fibronectin-containing complex which inhibits the expression of bovine leukemia virus p24. J. Virol. 79, 8164–8170.
Verma, S., 2007. Development of immunoglobulin diversity at the heavy chain locus of cattle. PhD thesis. University of Glasgow.
Zandomeni, R.O., Carrera-Zandomeni, M., Esteban, E., Donawick, W., Ferrer, J.F., 1992. Induction and inhibition of bovine leukaemia virus expression in naturally infected cells. J. Gen. Virol. 73 (Pt 8), 1915–1924.
Zandomeni, R.O., Esteban, E., Carrera-Zandomeni, M., Ferrer, J.F., 1994. Host soluble factors with blocking and stimulating activity on the expression of the bovine leukemia virus. J. Infect. Dis. 170, 787–794.