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Expression of bovine leukaemia virus (BLV) gp51 protein in blood and milk cells of cows with leukosis

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Abstract

Introduction: Bovine leukaemia virus (BLV) is the retroviral causative agent of enzootic bovine leukosis, the most common neoplastic disease of cattle and a serious problem worldwide. Its diagnosis is commonly by tests for antibodies recognising the p24 capsid protein and structural glycoprotein (gp) 51. With flow cytometry recently having come to veterinary immunology, applications for it may now include BLV. The study determined BLV gp51 expression in blood and milk lymphocytes of naturally infected cows by flow cytometry. **Material and Methods:** Nineteen Polish Black and White Lowland breed cows aged 4–9 years and naturally infected with BLV and ten uninfected counterparts had blood and milk sampled and cultured. The immunological status of the animals was confirmed with ELISA and PCR. Dual-colour flow cytometry analysis was performed with specific monoclonal antibodies for lymphocyte cluster of differentiation (CD) markers and gp51 viral envelope protein and conjugates labelled with fluorescein isothiocyanate or phycoerythrin. Bovine leukaemia virus gp51 was confirmed in lymphocytes by immunofluorescence with anti-gp51 monoclonal antibodies. **Results:** The gp51 antigen was detected in blood and milk lymphocytes of infected cows, but the percentage of cells expressing it in milk was much lower than in blood. A depleted number of CD4+ lymphocytes, an augmented number of CD8+ lymphocytes, a lower ratio of CD4+ to CD8+ and a proliferation of CD19+ immunoglobulin M+ cells were also found. **Conclusion:** These proliferated cells were immature, gave no sign of a tendency to differentiation and were characterised by prolonged vitality.

Keywords: bovine leukaemia virus, gp51 glycoprotein, lymphocytes, flow cytometry.

Introduction

Bovine leukaemia virus (BLV) belongs to the *Retroviridae* family and *Deltaretrovirus* genus and causes enzootic bovine leukaemia. The disease occurs worldwide and is listed by the World Organisation for Animal Health (OIE) (32). Approximately one-third of cattle infected with BLV develop persistent lymphocytosis and this state is characterised by a polyclonal expansion of B-lymphocytes, but some animals are frequently asymptomatic in an aleukaemic state and are virus carriers. Only a small percentage (1–5%) of infected cattle develop malignant lymphoma, the clinical form of BLV infection, and do so after a long latency period. The virus is present in the circulating peripheral blood lymphocytes of infected cattle and can be transmitted both horizontally and vertically. Horizontally, the virus is spread by direct exposure to materials contaminated with BLV such as blood, plasma and semen and by veterinary activities such blood drawing and rectal examinations. Vertical routes include feeding with colostrum and *in utero* infection. In some areas, haematophagous insects (*Tabanidae*) can spread BLV in cattle herds under natural conditions (27). Viraemic status is rather short and can only be detected during the first 14 days after infection, but the expression of the viral antigens is difficult to detect because viral protein production is suppressed by cytokines. Interleukin (IL)-6 and IL-10 cytokines in particular have suppressing influence on viral protein production (18, 27, 30). These studies suggested that BLV caused disease because of changes in host factors. Frie *et al.* (13) investigated lymphocyte subsets in dairy cows naturally infected with BLV and found that these cows exhibited abnormal B and T cell phenotypes after primary and secondary exposures to keyhole limpet haemocyanin. They demonstrated that

both B and T cell immunities are disrupted in BLV-infected cows even after primary immune exposure.

It is known that infectious BLV particles and/or somatic cells infected with BLV can be transmitted to calves by milk, but it is thought to be a minor route of infection. BLV infection affects production efficiency and causes decrements of immunological status, and the sanitary and economic impact of infection is associated with hindered international movement of cattle. In many European countries, national programs controlling the spread of BLV infection and eradicating the disease exist, and because of these recommendations the cattle population is relatively free from BLV (25). In May 2017, Poland was recognised as free from BLV infection.

Flow cytometry (FC) is a technique for qualitative and quantitative assessment of multiple parameters of individual cells or particles in different complex cell suspensions. The technology is an integral component of research in the life sciences, particularly immunology, and is in common use for diagnostic and prognostic investigations in human oncology (specifically in haematooncological diagnosis). Recently, the flow cytometry technique has been integrated into veterinary oncology and immunology. Flow cytometry determination of BLV glycoprotein (gp) 51 expression in blood and milk lymphocytes of BLV-infected cows was the aim of the study.

Material and Methods

Animals. Investigations were performed on 19 Polish Black and White Lowland breed cows naturally infected with BLV at the age of 4–9 years. Uninfected cows were adopted as controls, and were 10 of the same breed and similar age.

Serological investigations. Blood and milk samples were collected from infected and control animals. The BLV infection in the samples was monitored by ELISA using commercial kits and PCR methods, the latter detecting proviral DNA according to the protocol of Kuckleberg *et al.* (19).

Blood cell preparation. For the haematological examinations and fluorescence-activated cell sorting (FACS) analysis, blood was collected into sterile tubes containing ethylenediaminetetraacetic acid-K2 as anticoagulant. Total blood white cells and lymphocytes were counted and Schilling's formula was applied.

Cell culture of blood lymphocytes. To enable the expression of bovine leukaemia virus gp51 antigen, blood cells of BLV-positive and BLV-negative animals were grown in short-term cell cultures. Briefly, blood samples were layered on a 1.077 g/mL Histopaque gradient and centrifuged for 30 min at 600 × *g* at 4°C. Cells were harvested from the interphase and after three washings were counted and suspended at a concentration of 1×10^6 cells/mL in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated foetal calf serum and Antibiotic-Antimycotic Solution for cell culture containing 10,000 U penicillin, 10 mg streptomycin and 25 µg of amphotericin B per mL (Sigma-Aldrich, St. Louis, MO, USA). Concanavalin A (Con A; Sigma-Aldrich) was added to the medium at a concentration of 5 µg/mL. Cells were cultured in plastic Petri dishes for 48 h at 37°C and in an atmosphere of 5% CO_2. After incubation, the cell pellet was tested by flow cytometry and immunofluorescence (IF).

Preparation of cells from milk. Milk samples were centrifuged for 30 min at 700 × *g* at 4°C, the supernatant was discarded and the cell pellet was placed in 5 mL of phosphate-buffered saline (PBS). This procedure was performed twice, then the cells of the pellet were mixed in 1 mL of PBS and layered on 3 mL of a 1.080 density gradient composed of 24 parts 11.6% Ficoll 400 and 10 parts 30% Uropoline. The samples were centrifuged for 50 min at 900 × *g* at 4°C, then the cells from the interphase were collected and after three washings in PBS were cultivated *in vitro* under the same conditions as the blood lymphocytes. The cells were then harvested and analysed in a flow cytometer and by immunofluorescence.

Flow cytometry analysis. Lymphocyte subpopulations were analysed using the dual-staining method and a panel of monoclonal antibodies (MAbs) (VMRD, Pullman, WA, USA), and fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labelled conjugates (Medac, Wedel, Germany). Monoclonal mouse anti-bovine antibodies detecting bovine CD19+, CD4+, CD8+, CD19+ immunoglobulin M (IgM) and gp51 of BLV were used. Briefly, for FACS analysis, 50 µL of lymphocytes was incubated for 30 min at room temperature with 1 µL of primary MAbs, washed and then incubated for 30 min with goat anti-mouse immunoglobulin G (IgG + IgM) FITC-labelled conjugate in darkness. After washing, the cells were coupled with the secondary MAbs and incubated as previously. Next, the cell solution was washed, goat anti-mouse (IgG + IgM) PE-labelled conjugate was added and the cells were incubated again. Red blood cells were lysed in FACS Lysing Buffer (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were washed, fixed in a buffer containing formaldehyde and analysed in a FACSCalibur flow cytometer (Becton Dickinson) with an argon excitation source. Percentages of B and T cells were determined by Simulset and Lysys software (both from Becton Dickinson).

Immunofluorescence. Smears of cultured lymphocytes were air dried and fixed in cold acetone for 10 min at 4°C. Mouse anti-gp51 of BLV MAb (VMRD) diluted 1:100 was dropped onto the slides and these were incubated for 30 min at 4°C in a humid atmosphere in darkness. After three washings, the slides were covered with FITC-labelled goat anti-mouse immunoglobulin heavy and light chains monoclonal antibody diluted to 1:300 and incubated as

mentioned above. Then the slides were washed, buffered glycerol was dropped onto them and immunofluorescence was analysed in a fluorescence microscope (Olympus Life Science, Tokyo, Japan).

Statistical evaluation. Statistical analyses were performed with STATISTICA 10 software (StatSoft Inc., Tulsa, OK, USA). Data were analysed by Student's *t*-test and the level of significance was set at $P < 0.05$ in all cases. The independent-samples *t*-test was applied to compare the means between two relevant groups. Multiple box-and-whisker plots with sample median and quartile measures were created to illustrate a measure of the central tendency of peripheral blood lymphocyte **(**PBL) percentages in the aleukaemic (AL) and persistently lymphocytotic (PL) stages of infection**.**

Results

All cows were serologically positive and the results of molecular investigations showed the presence of proviral DNA. On the basis of haematological analysis, the animals fell into two groups: in eleven cows the lymphocyte levels were in the range of 7,600–11,200 cells per mm^3 (mean value 9,354 cells per mm^3) and these cows were classified as AL, and in the other eight cows very high lymphocyte values were determined in the range of 12,800–63,200 cells per mm^3 (mean value 29,650/mm^3) and the animals were classified as PL.

The results of flow cytometry determination of peripheral blood subpopulations are presented in Table 1.

On the basis of flow cytometry analysis, it was found that the expression of BLV gp51 in infected cattle was mainly in CD19+ and CD19+ IgM+ lymphocytes. In the AL stage of the disease, a lower percentage of CD19+ (58.3%) and CD19+ IgM+ (47.5%) B lymphocytes was found than in the PL stage (71.1% and 68.6% respective mean values).

The opposite dependency was demonstrated for CD4+ and CD8+ T lymphocytes: their higher percentages (22.1% and 13.5%, respectively) were found in the AL stage of infection and their lower ones (11.4% and 6.5%, respectively) in the PL stage (Fig. 1). A detailed analysis of the infection stage effect on the differentiation of the subpopulation sizes of PBLs revealed a significantly lower number ($P = 0.005$ and $P = 0.002$) of CD19+ and CD19+ IgM+ B lymphocytes as well as a negligibly lower number (not statistically confirmed, with $P = 0.348$ and $P = 0.743$) of CD4+ and CD8+ T lymphocytes in animals in the aleukaemic stage, compared to animals with persistent lymphocytosis.

The ratio of CD4+ to CD8+ T lymphocytes was lower in aleukaemic animals (2.80) than in persistently lymphocytotic animals (2.84). However, the difference between these values was not confirmed statistically ($P = 0.432$). The ratio of CD19+ to CD19+ IgM+ B lymphocytes was not significantly different for cows in the AL stage (1.66) from this ratio in animals in the PL stage (1.11) ($P = 0.363$). Thus, an increase in the number of CD19+ IgM+ cells coincided with an increase in total B lymphocytes (Tables 1 and 2).

Table 1. The percentage of peripheral blood lymphocyte subpopulations of bovine leukaemia virus–infected cattle in the aleukaemic (AL) and persistently lymphocytotic (PL) stages

Cow sample	ELISA	PCR	Phase of infection	Lymphocytes/mm^3	CD19+	CD19+ IgM+	CD4+	CD8+
1	+	+		7,600	25	46	25	3
2	+	+		7,800	67	75	15	17
3	+	+		8,400	54	80	27	23
4	+	+		8,800	45	27	31	10
5	+	+		9,000	54	45	26	32
6	+	+	AL	9,600	54	61	23	27
7	+	+		9,600	67	64	33	20
8	+	+		10,200	47	18	30	6
9	+	+		10,300	82	68	1	2
10	+	+		10,400	96	20	2	2
11	+	+		11,200	51	19	30	4
Mean value				9,354	58.3	47.5	22.1	13.5
1	+	+		12,800	66	65	21	4
2	+	+		15,200	55	77	27	31
3	+	+		15,600	39	62	13	5
4	+	+	PL	26,000	83	81	6	2
5	+	+		26,400	61	81	5	2
6	+	+		39,000	96	61	8	2
7	+	+		39,000	73	36	7	2
8	+	+		63,200	96	86	4	4
Mean value				29,650	71.1	68.6	11.4	6.5

CD – cluster of differentiation; IgM – immunoglobulin M

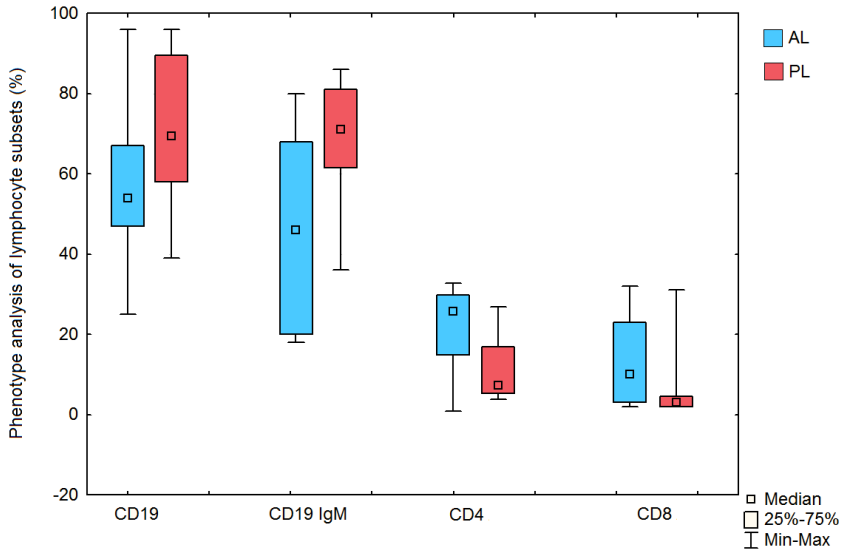

Fig. 1. The difference in the percentages of cluster of differentiation CD19+ and CD19+ immunoglobulin M+ B and CD4+ and CD8+ T lymphocyte subpopulations depending on bovine leukaemia virus infection stage
AL – aleukaemic stage of infection; PL – persistently lymphocytotic stage of infection. Median values were used to provide a better measure of the central tendency

Table 2. The expression of gp51 in blood lymphocyte subsets of bovine leukaemia virus infected cattle (%)

Cow sample	Stage of infection	Lymphocytes/mm^3	CD markers + gp51 (%)				Cells with gp51 expression
			CD19+	CD19+ IgM+	CD4+	CD8+	
1	AL	7,600	15	20	0	16	51
2		7,800	25	7	1	7	40
3		8,400	29	20	0	10	59
4		8,800	38	12	2	10	62
5		9,000	30	18	3	21	72
6		9,600	38	13	0	17	68
7		9,600	1	3	0	2	6
8		10,200	24	10	0	1	35
9		10,300	3	3	0	0	6
10		10,400	28	19	3	12	62
11		11,200	1	1	0	0	2
Mean value		9,354	21.0	11.4	0.8	8.7	41.3
1	PL	12,800	16	10	1	4	31
2		15,200	21	24	0	17	62
3		15,600	19	30	0	5	54
4		26,000	31	9	0	2	42
5		26,400	9	8	1	5	23
6		39,000	34	23	2	2	61
7		39,000	28	18	0	2	58
8		63,200	68	20	1	4	93
Mean value		29,650	28.25	17.7	0.6	5.2	53.0

CD – cluster of differentiation; IgM – immunoglobulin M; AL – aleukaemic; PL – persistently lymphocytotic

Additionally, the B cell to T cell ratios were lower in aleukaemic cattle (10.5) than in animals in the PL stage (16.4). These differences between ratios were caused by an excessive proliferation of immature CD19+ IgM+ cells and a fall in the CD8+ T lymphocyte percentage (Fig. 1).

In the PL stage of infection, strong proliferation and accumulation of B lymphocytes and expression of both CD19+ and CD19+ IgM+ markers were detected. The association of these two markers as well as CD4+ and CD8+ markers in PBLs obtained from BLV-infected cows and the expression of BLV gp51 antigen in these cells were examined in two-colour flow cytometry and the results are presented in Table 2. The percentage of CD19+ B cells which expressed BLV gp51+ antigen depended on the progress of BLV infection and increased from 21.0% in the AL to 28.25% in the PL stage. Similarly, the percentage of CD19+ IgM+ B cells with BLV gp51+ antigen expression increased from 11.4% in the AL to 17.7% in the PL stage of infection (Table 2). An opposite tendency was observed with BLV gp51+ antigen expression in CD4+ and CD8+ T cells, the higher percentages of which (0.8% and 8.7%, respectively) were noted in the AL stage of infection and the lower values (0.6% and 5.2%, respectively) in the PL stage (Figs 2, 3A and 3B).

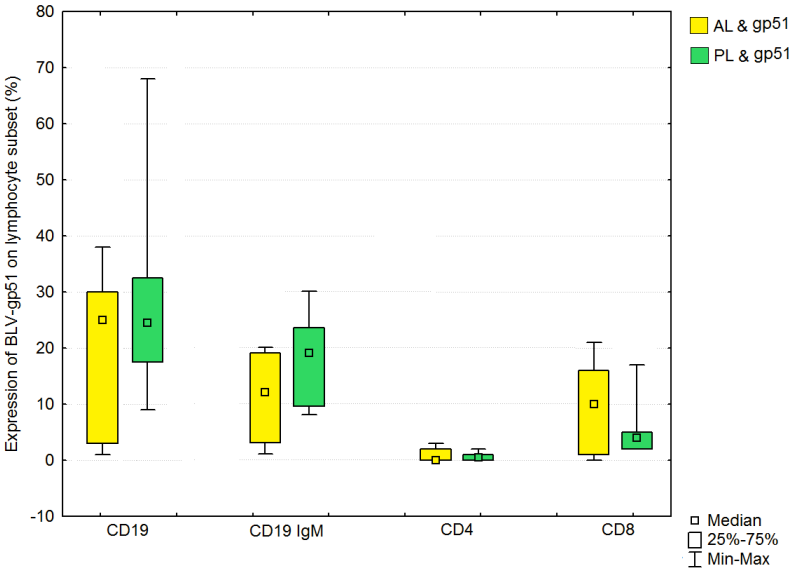

Fig. 2. The effect of BLV gp51 antigen expression in the of cluster of differentiation CD19+ and CD19+ immunoglobulin M (IgM)+ B and CD4+ and CD8+ T lymphocyte subpopulations
AL – aleukaemic stage of infection; PL – persistently lymphocytotic stage of infection

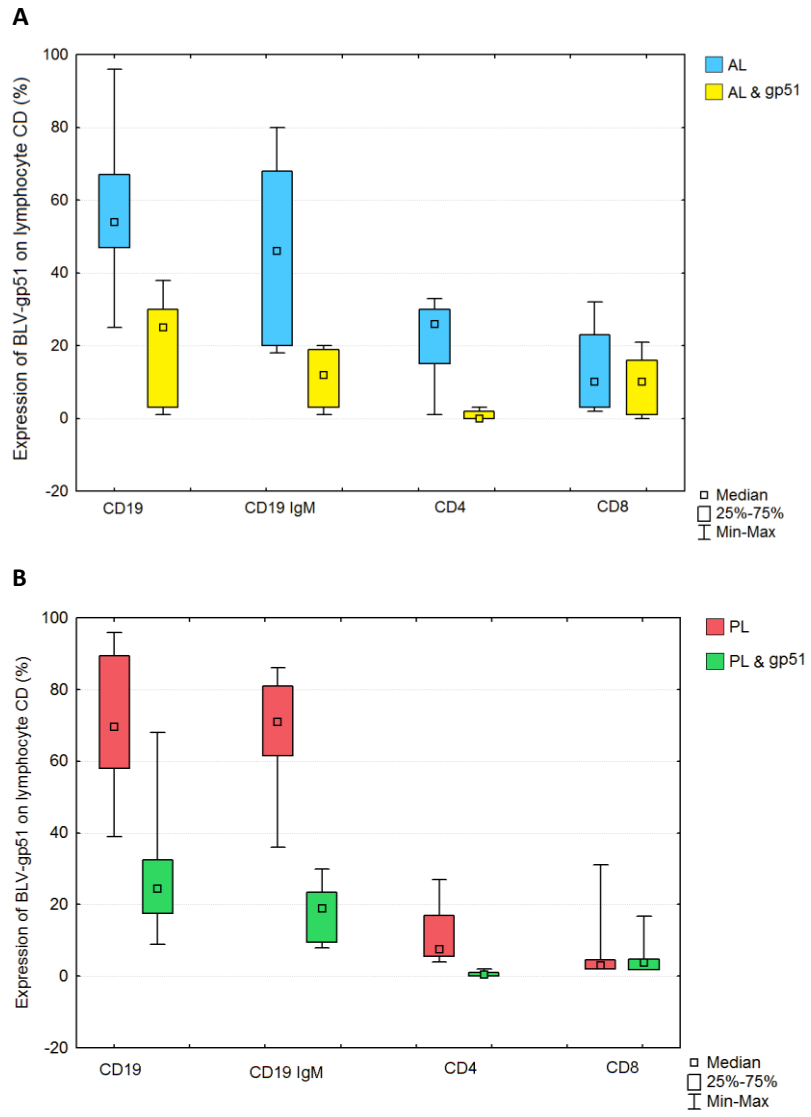

Fig. 3. The effect of BLV gp51 antigen expression in the number of cluster of differentiation CD19+ and CD19+ immunoglobulin M (IgM)+ B and CD4+ and CD8+ T lymphocyte subpopulations
AL – aleukaemic stage of infection; PL – persistently lymphocytotic stage of infection

An analysis of the presence of the gp51 antigen in blood lymphocytes revealed a significantly lower number of CD4+ T cells expressing it in the aleukaemic stage (76/mm^3, 0.8%) ($P < 0.003$ and $P < 0.004$, respectively) compared to the numbers of CD19+, CD19+ IgM+ and CD8+ cells expressing the gp51 antigen in this stage (1,918/mm^3, 21.0%; 1,034/mm^3, 11.4%; and 777/mm^3, 8.7% respectively). The analysis also discovered a significantly lower number of CD4+ gp51+ cells in the PL stage of infection (226/mm^3, 0.6%) ($P < 0.008$ and $P < 0.03$, respectively) in comparison with the numbers of B and CD8+ cells expressing this antigen in this stage (10,726/mm^3, 28.25%; 5,336/mm^3, 17.7%; and 1,226/mm^3, 5.2% respectively) (Fig. 3A and B). It was observed that the weakening of the expression of CD4+ gp51+ lymphocytes in peripheral blood correlated with the fall in the CD4+ gp51+ to CD8+ gp51+ ratio. Long-term infections showed a stronger contraction of the CD4+ gp51+ and CD8+ gp51+ lymphocyte subsets that also included a decrease in the absolute size of the CD4+ cell population and the absolute number of CD8+ cells. The results concerning the expression of the gp51 antigen in cells from milk are presented in Table 3 and a comparison of this expression in blood and milk is illustrated in Fig. 4. The expression of gp51 in CD19+ and CD19+ IgM+ cells from milk in the AL stage was low: the respective mean values were 3.5% and 1.8%. In animals remaining in the PL stage, these values increased and the maximal percentages found in persistent lymphocytosis for CD19+ and CD19+ IgM+ were 6.3% and 2.7%, respectively.

Table 3. The expression of gp51 in milk lymphocyte subsets of bovine leukaemia virus–infected cows (%)

Cow sample	Stage of infection	Lymphocytes/mm^3	CD markers (%)						Expression of gp51 in milk lymphocytes (%)
			ELISA	PCR	CD19+	CD4+	CD8+	CD19+ IgM+	
1	AL	7,600	+	+	1	–	–	1	2
2		7,800	+	+	2	–	–	–	2
3		8,400	+	+	–	–	–	3	3
4		8,800	+	+	–	–	–	2	2
5		9,000	+	+	2	–	1	–	3
6		9,600	+	+	1	–	1	–	2
7		9,600	+	+	1	–	–	3	4
8		10,200	+	+	1	–	1	1	3
9		10,300	+	+	3	–	–	–	3
10		10,400	+	+	18	–	–	4	21
11		11,200	+	+	10	–	–	6	20
Mean value		9,354	+	+	3.5	-	0.3	1.8	5.7
1	PL	12,800	+	+	1	–	–	1	2
2		15,200	+	+	3	–	–	2	5
3		15,600	+	+	1	–	–	2	3
4		26,000	+	+	2	–	1	–	3
5		26,400	+	+	9	1	2	3	15
6		39,000	+	+	4	–	–	3	7
7		39,000	+	+	10	–	1	2	13
8		63,200	+	+	21	–	4	9	33
Mean value		29,650			6.3	0.1	1.0	2.7	10.2

CD – cluster of differentiation; IgM – immunoglobulin M; AL – aleukaemic; PL – persistently lymphocytotic

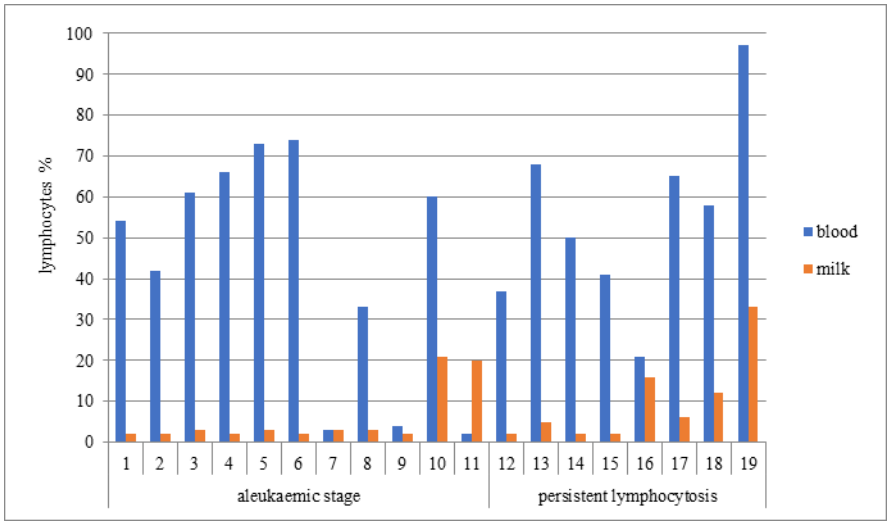

Fig. 4. The expression of glycoprotein 51 in blood and milk lymphocytes of bovine leukaemia virus-infected cows with clearly much higher expression in blood lymphocytes than in milk cells

In animals in the AL stage, gp51 expression was determined in a mean 41.3% of blood lymphocytes. The highest mean values of gp51 expression were determined in CD19+ lymphocytes at 21.0% and CD19+ IgM+ lymphocytes at 11.4%. Cells of CD4+ type expressed the antigen in a mean 0.8% of their population and CD8+ lymphocytes did so in 8.7% of cells (also the mean value).

The presence of gp51 in milk lymphocytes of animals in the AL stage was detected in 2%–21% of cells, the mean value being 5.7%. This glycoprotein was present in 3.5% of cells with CD19+ antigen and in 1.8% of CD19+ IgM+ lymphocytes. Glycoprotein 51 was absent from CD4+ lymphocytes, and only in low presence in CD8+ lymphocytes, where 0.3% of cells had this protein detectable. In milk samples of cows in the PL stage of leukaemia, the presence of gp51 in milk was detected in 2%–33% of milk lymphocytes for a mean value of 10.2%. The expression of gp51 was detected in 6.2% of CD19+ and in 2.7% of CD19+ IgM+ lymphocytes. In CD4+ T cells, this expression was found in 0.1% and in CD8+ T lymphocytes in 1.0% (Fig. 5A and 5B).

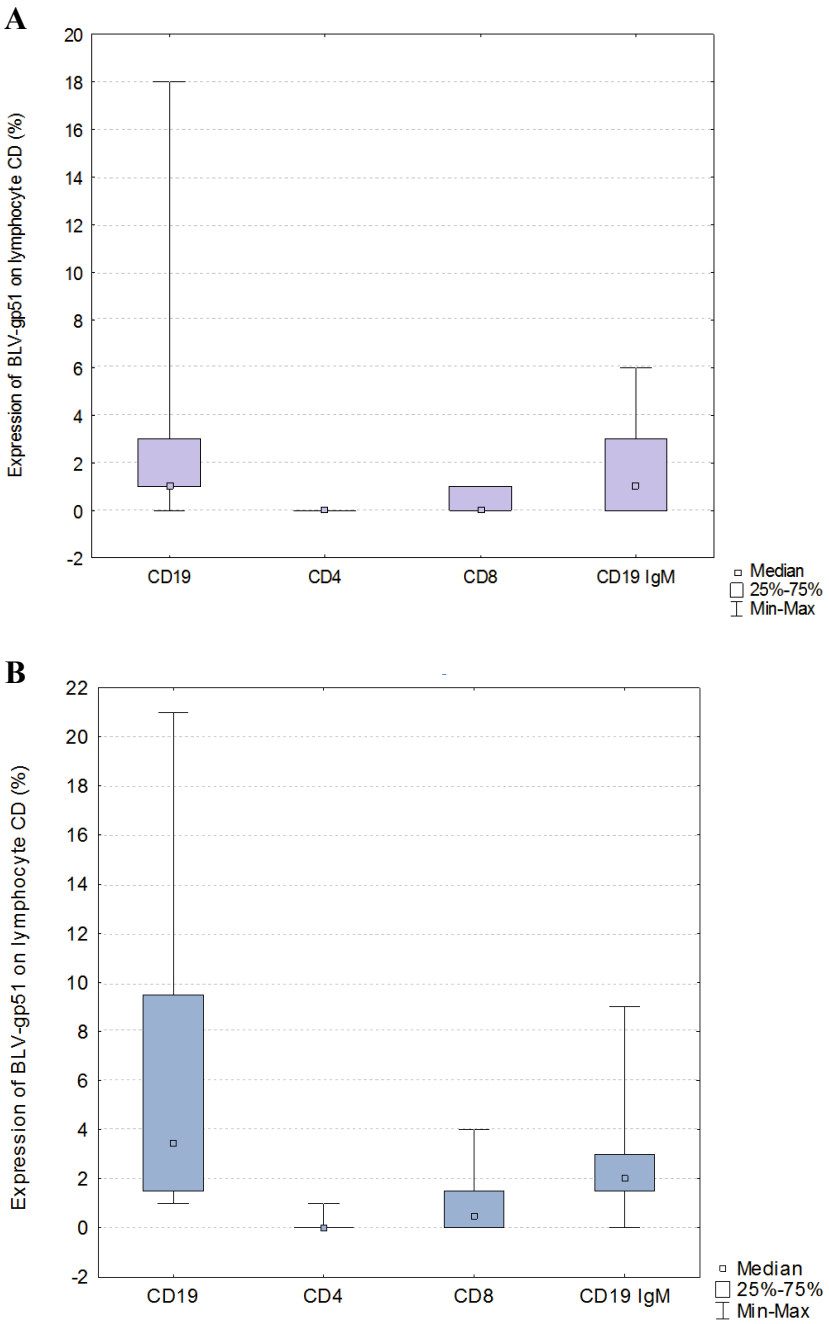

Fig. 5. The expression of BLV glycoprotein (gp) 51 antigen in milk: in cluster of differentiation CD19+, CD19+ immunoglobulin M (IgM)+ B and CD4+, CD8+ T lymphocyte subpopulations
A – AL stage of infection; B – PL stage of infection

The highest gp51 expression was determined in milk lymphocytes of cows with high-count lymphocytosis: there were 21% of CD19+, 9% of CD19+ IgM+ and 4% of CD8+ cells. The results indicated that CD19+ and CD19+ IgM+ lymphocytes are the main target cells, because this was where BLV gp51 expression was predominantly detected. These results are presented in Table 3 and Fig. 4. The presence of green fluorescence in smears stained with conjugate attached to anti-gp51 monoclonal antibodies confirmed the presence of BLV gp51 on blood and milk lymphocytes (Figs 6 and 7).

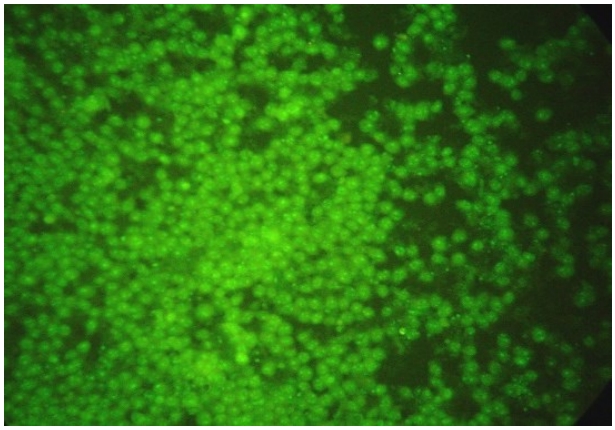

Fig. 6. Immunofluorescence showing the expression of bovine leukaemia virus glycoprotein 51 in blood lymphocytes of a cow with persistent lymphocytosis

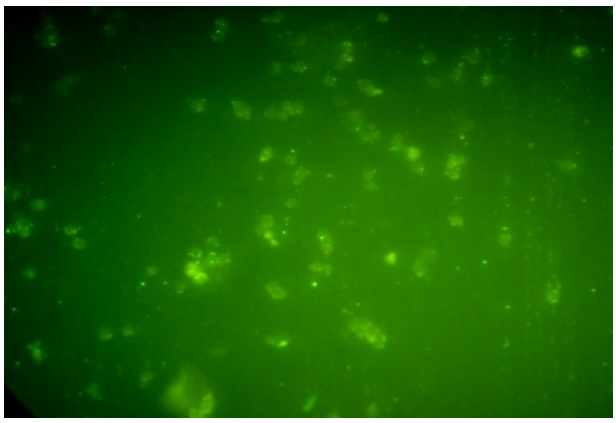

Fig. 7. Immunofluorescence showing the expression of bovine leukaemia virus glycoprotein51 in cells from a milk sample from a leukaemic cow

Discussion

The results of our investigations indicated that the expression of BLV gp51 was present in blood and milk lymphocytes of animals infected with BLV. The gp51 expression was much higher in lymphocytes from blood than in those from milk. The studies performed by Bartlett *et al.* (4) confirmed that infectious BLV and/or BLV-infected cells were present in colostrum and milk from most BLV-infected cows. They examined the role of colostrum and milk in the natural transmission of BLV and the development of leukaemia in calves which were fed from birth with colostrum and milk from their BLV-positive mothers and kept in complete isolation from infected cattle. Most of these calves remained healthy and BLV was detected in only 6 of the 41 partially or completely isolated calves that ingested colostrum and milk from their BLV-infected mothers. Their results showed that milk-borne transmission plays a secondary role in the natural spread of BLV, and that milk and colostrum from most BLV-infected cattle contain cells positive for the virus. It has been reported that feeding infected bulk milk can initiate infection in neonatal animals. There are recommendations by the World Organisation for Animal Health to feed calves with milk from healthy cows or with milk replacements (32).

Our results showed that gp51 expression in blood lymphocytes of cows in the AL stage of bovine leukaemia was detected mainly in CD19+ and CD19+ IgM+ lymphocytes, which indicated BLV tropism for B cells and underlined the role of B lymphocytes in BLV infection. The gp51 antigen was also detected in T cells, but the expression was not strong, which confirmed that T cells can also be the source of viral particles. Meirom *et al.* (21) investigated cows in the PL stage of BLV infection with three-colour cytometric analysis and found that at this stage the leukaemia virus caused strong proliferation of B cells with expression of both the markers IgM and CD5 and association with BLV gp51 expression in these cells. Our results are in agreement with those obtained by these authors.

Bovine leukaemia virus induces abnormal B cell proliferation and B cell lymphoma in cattle, where the BLV provirus is integrated with the genome of the host. BLV-infected B lymphocytes rarely express viral proteins *in vivo*, but short-term cultivation evidently induces BLV expression in some, but not all, BLV-infected B cells as found by Ikebuchi *et al.* (16). They showed that the number of IgM^{high} B cells increased in the blood of BLV-infected cattle and that they were likely to express BLV antigens after *ex vivo* cultivation. It suggests their role as sources of BLV infection. On the other hand, IgM^{low} B cells included a high proportion of BLV-silencing cells, which expressed high levels of some protooncogenes (16).

The expression of gp51 in blood and milk lymphocytes of cows with persistent lymphocytosis was much stronger than in aleukaemic animals. This expression was visible predominantly in CD19+ and CD19+ IgM+ lymphocytes. The obtained results indicated the role of milk in BLV transmission in the herds. Although BLV demonstrates tropism for B cells, it can affect both adaptive and innate immunities because these systems share many effector mechanisms. Mammary gland immunity is largely dependent upon neutrophilic functions. Many studies have investigated the effects of BLV infection on lymphocyte subsets in infected animals (9, 29, 30), B cell vitality (11, 28, 31) and neutrophil functions (10,

28). Bovine leukaemia virus can be the reason for changes in milk production and fertility in cows. Many authors indicated its negative economic impact on cattle farming. Mastitis and decreased milk production have been associated with BLV infection, particularly in BLV-infected animals with persistent lymphocytosis and on high-performing infected dairy farms (11, 35). Infection with BLV caused economic losses not only in its effect on milk production but also in impairing animal reproduction, which was indicated by many researchers (33, 35); however, in previous investigations other authors found no differences in milk production by BLV+ and by BLV− cows.

Investigations in Canada by Nekouei *et al.* (23) offer much detailed information concerning the longevity of and milk production by leukaemic cows. The results indicated the potential causal associations between BLV infection and lifetime production and longevity. BLV-infected cows had a consistently shorter lifespan than BLV-free animals. This suggested that the infection could be one of the main causes of premature culling (or premature death in some cases) of dairy cows. It was predicted that test-positive cows produced substantially lower milk yields than BLV-negative ones during their lifespan. On the other hand, it has been suggested that cows with higher milk production potential may be more susceptible to BLV infection. In contrast to both theories, there have been other studies which could not prove any significant association between BLV infection and the measures of production (33). Infection with BLV caused monocytes from BLV-infected cows with persistent lymphocytosis to release the greatest amount of hydrogen peroxide (2). However, phagocytes producing a large quantity of reactive oxygen species (ROS) are essential for killing bacteria, and an excess level of ROS production over time can lead to oxidative stress, which can contribute to the pathogenesis of BLV infection. In BLV-infected dairy cows with persistent lymphocytosis, inhibition of monocyte and neutrophil apoptosis was observed and the percentage of neutrophils producing ROS was lower in these animals, while ROS production by monocytes was more intensive. Additionally, in cows in the PL stage, upregulation of B cells with the CD markers CD5+ and CD11b+ was observed, which was the result of the apoptosis inhibition (5). The investigations of Souza *et al.* (28) showed that BLV infection was associated with a great decrease in the antioxidant enzymes glutathione peroxidase and superoxide dismutase. Della Libera *et al*. (10) indicated that milk leukocytes in BLV-infected cows mainly in the PL stage of infection are functionally altered, which might impact the outcome of udder infections. Yoshikawa *et al.* (35) investigated serum and milk antibody titres against the BLV gp51 antigen and found that they were higher in serum than in milk. These results agree with our finding that gp51 expression was much higher in blood cells than in milk. These authors also examined iron metabolism in BLV-infected dairy cows through iron and ferritin levels in serum and milk. Iron and ferritin concentrations were significantly higher in blood serum than in milk, but they also found that the ferritin concentrations in the milk of the infected cows were significantly lower than those in the milk of normal control cows. The titres of anti-BLV antibody in the milk samples correlated highly with iron concentrations in the serum. These data suggest that infection with BLV affects iron homeostasis through iron metabolism in the cow mammary gland (33). Bovine leukaemia virus infection may activate the cytokine receptor and B cell growth cytokines (IL-4, IL-6 and IL-10) (18, 30).

Infection with BLV also has been shown to alter the expression of other cytokines besides IL-4, IL-6 and IL-10, namely IL-2 and IL-12. The virus' regulation of several immunomodulators provides multiple mechanisms for it to suppress immunity in affected cattle (14). Interleukin 6 may play a contributory role in BLV latency due to its elevation in blood (14, 30). Additionally, IL-6 mediates hypoferraemia developing in inflammation through reduction of hepcidin, which reduces intestinal iron absorption and releases iron from macrophages (24). High ferritin concentrations were detected in sera of BLV-infected cows (26). Ferritin can be a tumour marker in humans and a useful factor to monitor treatment effectiveness (1).

Many authors investigated the relationship between BLV and tumours in humans and the possibility of human infection with BLV after milk and meat consumption or in association with work on cattle farms or abattoirs. Maruyama *et al.* (20) demonstrated that BLV and HTLV-I are capable of evoking a cross-reactive immune response in at least some hosts in natural infection as well as by virus vaccination.

Bovine leukaemia virus capsid proteins were detected in human serum by Buehring *et al*. (6, 7, 8) and they indicated that humans have antibodies which react with BLV. Gene sequences of BLV were identified in human breast cancers also by Buehring *et al.* (6) using PCR. They found that 67 out of 114 women's breast cancer samples were positive for BLV, and this compared with 30 out of 104 normal breast controls. A similar prevalence pattern was detected by Baltzell *et al.* (3). They investigated the role of multiple oncogenic viruses, among them mouse mammary tumour virus, bovine leukaemia virus, human papilloma virus, and Epstein–Barr virus, and identified them as separate infectious pathogens in human breast cancer. The authors demonstrated that these four viruses may be present in normal breast tissue and benign tumours 1 to 11 years before the development of breast cancer in the same patients.

On the other hand, other investigations by PCR did not find BLV in breast cancer in China. With the use of whole-genome sequencing of DNA from 51 breast cancer tissue samples, Gillet *et al.* (15) were able to state that breast tumours are devoid of bovine

leukaemia virus DNA, and their results excluded the possibility of a clonal insertion of BLV into breast tumour cells and strongly argued against an association between BLV and breast cancer.

The results of investigations performed by Yamada *et al.* (34) indicated the role of exosomes in BLV infection development. Exosomes are very small membranous particles 40–100 nm in diameter. In humans they are present in blood, plasma and other physiological body fluids including breast milk. These microvesicles contain mRNA, microRNA (miRNA) and intracellular proteins. Some authors suggested that the exosomes play a role in intracellular communication: that it may be possible through direct interaction of exosomal surface antigens with target cell receptors or by transport of RNA and proteins to target cells from exosomes. It has been reported that exosomes released from virus-infected cells contain viral nucleic acids and proteins, which was demonstrated in both RNA and DNA infections in humans infected with HIV, herpes simplex, Epstein–Barr, hepatitis C viruses and *Leishmania* spp. These exosomes are considered to be engaged in viral infection and pathogenesis and can be possible biomarkers of diseases, especially in the diagnosis and prognosis of cancers.

There are many active molecules such as growth factors and cytokines present in bovine milk and as many as 2,107 proteins in bovine milk exosomes have been identified. It is evident that bovine milk exosomes play important roles in the growth of infants, such as driving immune system maturation and responsiveness. Zhou *et al.* (36) identified 602 miRNAs originating from 452 miRNA precursors (pre-miRNAs) in exosomes of human breast milk with the use of deep sequencing. They found that 867 well-characterised immune-related pre-miRNAs were present in breast milk exosomes and these endogenous immune-related miRNAs were more resistant to extreme conditions. These authors speculated that these exosomal miRNAs are transferred to the infant from the mother *via* the digestive tract and are indispensable to the development of young immune systems.

Bovine milk exosomes may be useful and serve as biomarkers of the physiological state and infectious diseases. Yamada *et al.* (34) isolated exosomes from both BLV-infected cows and BLV-infected culture cells and they showed the presence of BLV enzyme reverse transcriptase and BLV gp51 and p24 structural proteins. These exosomes seemed to be non-infectious to cells or were low in infectivity. Moreover, the BLV proteins detected in bovine milk exosomes were reported to correlate with the presence of anti-bovine leukaemia virus antibodies in milk. Cows infected with BLV may release bovine leukaemia virus particles or its proteins *via* milk, which can provide a potential mechanism of BLV transmission.

Infection with BLV is correlated with the inhibition of apoptosis, and leads to the generation of a reservoir of infected cells. This circumstance and BLV tropism for B lymphocytes determined in infected animals may explain the lower number of milk B cells, especially in the PL stage, which were undergoing apoptosis. This phenomenon was observed in blood B cells by many authors (11, 14). These studies indicate the importance of monitoring and eradication of BLV infections in cattle, especially for human health and the minimisation of economic losses.

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