POLYMORPHISM OF \( \alpha \)-LACTALBUMIN GENE AND CHANGES OF THE PROFILE OF PERIPHERAL BLOOD LYMPHOCYTES IN COWS NATURALLY-INFECTED WITH BOVINE LEUKAEMIA VIRUS

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Received for publication June 06, 2006.

Abstract

Studies were performed on a population of 71 Black-and-White breed cows, aged 3-6 years, from a leukosis prevalent herd. Polymorphism of the \( \alpha \)-LA gene in the -1689 position was assayed with the PCR-RFLP method using Sdu I enzyme. The profile of peripheral blood lymphocytes was determined with the use of immunofluorescence technique with monoclonal antibodies. Associations between \( \alpha \)-LA gene polymorphism and the profile of peripheral blood lymphocytes were found. They concerned mainly the CD4+ and CD8+ T lymphocytes. Moreover, a significant effect of BLV infection on the number of CD19+ B lymphocytes and the percentage of CD2+, CD4+ and CD8+ T lymphocytes was recorded. The effect of the \( \alpha \)-LA gene polymorphism and the BLV infection on the profile of the peripheral blood lymphocytes was modified by the interaction of these two factors. It was exhibited by a different profile of changes among the EBL+ and EBL- cows. Bovine leukaemia virus also modified the dynamics of changes in the percentage of B lymphocytes in the first three months after calving.

Key words: cows, bovine leukaemia virus, \( \alpha \)-lactalbumin gene, lymphocytes, genetic polymorphism.

The \( \alpha \)-lactalbumin (\( \alpha \)-LA) gene occurs in cattle on the BTA 5 q21 chromosome (17). It consists of 4 exons and 3 introns and its length reaches 2023 bp. This gene is highly homologous with \( \alpha \)-LA genes of other mammalian species (18). In European dairy cattle breeds polymorphism has been observed so far only at positions +15, +21, +54 (4), +103 (30) and at position – 1689 of the 5' flanking region (39). Variability in this gene region can affect the ability to bind RNA polymerase and transcription factors and, as a result, can produce a different expression level of mRNA encoded by this gene (4, 39). The \( \alpha \)-LA gene product is a small globular protein which, in the majority of mammals, is built of 123 amino acids. The role of \( \alpha \)-LA in lactose biosynthesis and milk secretion has been generally studied. However, the role of \( \alpha \)-LA in immunological processes has been insufficiently researched. It is believed that it has an effect on the synthesis of the \( 1\beta \)-interleukin (IL-1\( \beta \)) (40) which stimulates the immunological response at many levels. This interleukin affects the T lymphocyte proliferation (15) and modifies the B lymphocyte proliferation and differentiation (21).

Recent study results indicate that the \( \alpha \)-LA participates in the process of apoptosis and in the counteraction of excessive proliferation of breast cancer cells in women (11, 16). It is not known, however, whether \( \alpha \)-LA also participates in the proliferation of tumorous cells in bovine leukaemia virus (BLV) infected cows. This virus is an etiological agent responsible for the development of enzootic bovine leukosis (EBL). This disease disturbs the B lymphocyte proliferation and development (31). In some BLV-infected animals an aleukaemic (AL) form is recorded in which the number of lymphocytes is regular or lower than in non-infected animals (3, 24). In approximately 30% of infected animals a chronic lymphocytosis can develop which is accompanied by changes in morphology and number of lymphocytes (5). It is presumed that the organism capacity to respond with lymphocytosis to BLV infection is an inherited character and exhibits connections with class II genes of BoLA system (43). A tumorous leukaemia can develop in 2-10% of the infected animals and it is characterized by tumorous lesions in various internal organs and is lethal to animals (6). Enzootic bovine leukaemia most frequently occurs in high yielding cows and contributes to significant economic losses. The mechanisms controlling the development of persistent lymphocytosis and lymphosarcoma have not yet been thoroughly researched.

The participation of \( \alpha \)-LA in inducing of apoptosis of breast cancer cells in women and the scarcely studied role of the protein in immunological processes...
and EBL pathogenesis in cattle prompted the authors to undertake studies with the aim of determining a relationship between α-LA gene polymorphism and the profile of peripheral blood lymphocytes in animals naturally infected with bovine leukaemia virus.

Material and Methods

Material and tests. The studies were performed on the population of 71 Black-and-White breed cows, aged 3-6 years, from a leukemia-prevalent herd. The animals were reared indoors in stables under good zoohygienic conditions. The herd was free from tuberculosis and brucellosis.

Diagnosis of enzootic bovine leukemia was confirmed by PCR and ELISA. The cows positive either in the ELISA or PCR were designated as EBL-positive, while those which gave a negative result in 3 subsequent months of lactation were designated as EBL-negative. Initial analyses were performed in the second half of the first month of lactation and were continued in the second and third month after calving in monthly intervals.

DNA isolation and PCR protocol. The genomic DNA was isolated from peripheral blood leukocytes with the Master Pure™ Purification Kit (Epicentre, USA) according to the procedure supplied by the producer. The amount (GeneQuant, Pharmacia) and the quality (electrophoresis) of DNA isolated was assessed. A fragment of a BLV gene with a length of 364 bp located in the area of gag gene and a fragment of milk kappa casein gene with a length of 273 bp were amplified from genomic DNA (an indicator of a proper course of PCR reaction). The primers (TIB-MOLBIOL, Poland) and the previously described PCR protocol were applied (22). The separation of the PCR products and the visual assessment of the results were completed according to the described procedure.

α-lactalbumin gene polymorphism. Polymorphism of the α-LA gene in the -1689 position was assayed with PCR-RFLP as described by Kamiński (23). The primers of the following nucleotide sequence were used:

forward: 5’ ATCAGTCTGGTTGGCTATT 3’
reverse: 5’ TATGCCAGGCTCATTCCTCT 3’
and a restriction enzyme Sdu I.

The A allele’s sequence of AGAGTTGGACACTACTGA is not recognized by this enzyme and, as a result, its length of 483 bp is alike the PCR product. On the other hand, the B allele has a cut off site for Sdu I and it is identified electrophoretically based on the DNA fragments of lengths: 263 bp and 220 bp.

Identification of peripheral blood lymphocytes. The profile of the peripheral blood lymphocytes was assayed with the use of immunofluorescence (IF) as described previously (22). The following primary antobody monoclonal antibodies (Mab) were used: anti-B-B2 (BAQ44A, IgM) (32) which bind on the lymphocyte surface with the B molecule similar to the CD19 in humans (41), anti-BoCD2 (MUC2A, IgG2α) (10), anti-BoCD4 (GC50A1, IgM) (8), anti-BoCD8 (CACT80C, IgG1) (32), and the antibodies that identify the WC1-N2 molecule (BAQ44A, IgG1) (8) occurring on the double negative T lymphocyte surface (CD4-CD8) (VMDR Inc. Pullman, USA). Additionally, secondary antibodies: goat anti-mouse IgG+IgM (H+L) conjugated with FITC, and goat anti-mouse IgG (H+L) labeled R-PE (Medac, Germany) were used. The control was prepared with the same protocol but without the addition of the first antibody. A fluorescence microscope (AxioLab, Zeiss, Germany) equipped with an appropriate filter sets was used for the cell identification. Counting was based on 200 cells registered in the visual field. Calculations were made to determine the absolute number and percentage of lymphocytes with a specified surface marker.

Statistical analysis. The statistical analysis included mean, standard deviation and data distribution fit tests with the normal distribution curve. In the absence of conformity with this model, the values of traits analysed were subjected to logarithmic transformation and statistical calculations were made on logarithmic values (log10). A three-factorial analysis of variance (General ANOVA /MANOVA) was applied. The impact of α-LA gene polymorphism (genotypes AA, AB, BB) (factor 1), BLV infections (EBL+; EBL-) (factor 2) and month of lactation (1, 2, 3 months after calving) (factor 3), as well as the impact of interactions between these factors on the level of the studied indices were analysed. Moreover, the effect of polymorphism of α-LA gene and the month of lactation as well as the effect of the interaction between these factors on the level of the studied indices were analysed. Furthermore, the effect of polymorphism of α-LA gene and the month of lactation as well as the effect of the interaction between these factors on the level of the studied indices were analysed in the EBL-positive and EBL-negative cows (two-factorial variance analysis). The significance of differences between the particular groups of animals was analysed based on the POST HOC TEST and Scheffe’s method. Calculations were made with STATISTICA 6.0 computer software.

Results

The applied diagnostic tests identified 54 leukemic (76.1%) and 17 clinically healthy cows (23.9%) in the examined herd. The study into the α-lactalbumin gene polymorphism revealed the α-LA^AA, α-LA^AB and α-LA^BB genotypes in the examined animals. The α-LA^AA genotype was found in 39 cows (54.9%), the α-LA^AB genotype in 26 cows (36.6%), while the α-LA^BB genotype was recorded in 6 animals (8.5%). Within the EBL-negative cows, 11 specimens (64.7%) represented the α-LA^AA genotype, 4 animals (23.5%) had the α-LA^AB genotype, and 2 cows (11.8%) exhibited the α-LA^BB genotype. Within the EBL-positive animals, the α-LA^AA genotype cows dominated with 28 cows (51.9%). The α-LA^AB genotype was found in 22 cows (40.7%), while α-LA^BB genotype occurred in 4 cows (7.4%).

Among the peripheral blood lymphocytes, the greatest population was represented by the CD19+ B lymphocytes (3.60x10^9/L; 39.2%) and the CD4+ T lymphocytes (3.0x10^7/L; 33.0%). The CD4+ T cells
(1.89x10^9/L; 20.8%) and the CD8+ T cells (1.22x10^9/L; 13.8%) constituted a smaller population and the T lymphocytes with TCRγδ (WC1-N2) formed the smallest subpopulation (0.79x10^9/L; 9.2%).

In the analysis of the effect of the α-LA gene polymorphism on the number and percentage of the CD19+ B lymphocytes, no significant differences were found between the genotypes (Table 1). However, significant differences were noted in relation to the T lymphocytes. The number and percentage of the CD4 lymphocytes were significantly higher in the α-LA^B/B homozygotes (α-LA^A/A and α-LA^B/B genotypes (P ≤ 0.01). Moreover, significant diversification in the absolute number of the CD2+ (P ≤ 0.01) and CD8+ (P ≤ 0.05) T lymphocytes was observed. The greatest numbers of these cells were also recorded in the α-LA^B/B homozygotes, whereas the lowest numbers were found in the homozygotes (α-LA^A/A) and heterozygotes (Table 1).

A similar variation between the genotypes was observed with reference to the number of the WC1-N2 T lymphocytes, however, these differences were not statistically significant (P=0.08). Moreover, a greater percentage of the CD2+ and CD8+ T lymphocytes was observed in the homozygotes α-LA^B/B, however, these differences were also not statistically significant (P=0.10 and P=0.19, respectively).

In the analysing of the effect of EBL on the profile of the peripheral blood lymphocytes, a significant (P≤0.05) diversification in the number of the CD19+ B lymphocytes was recorded between the EBL+ and EBL- cows (Table 1). The number of these cells was greater in the leukaemic cows than in the clinically healthy cows. Moreover, the percentage of the CD19+ B lymphocytes was higher, however, these differences were not statistically significant (P=0.20). The analysis of the quantitative changes of different T lymphocytes in EBL+ cows showed a significantly lower percentage of the CD2+ (P≤0.05), the CD4+ (P≤0.01) and the CD8+ (P≤0.01) T lymphocytes compared to clinically healthy animals, however, the absolute numbers of these cells were similar and the differences between them were statistically insignificant. On the other hand, the effect of the interactions between the α-LA polymorphism and the BLV infection on the number and percentage of the analysed lymphocytes (Figs 1a, 1b, 2a, 2b) was found. The correlation between these two factors caused a different quantitative diversity in the EBL-negative when compared with the EBL-positive animals. In the clinically healthy animals, significantly greater numbers of cells with CD19, CD2, CD4 and CD8 markers were recorded in the heterozygotes, while in the α-LA^A/A and α-LA^B/B homozygotes the number of these cells was lower and maintained at a similar level (Fig. 1a). A different trend of changes within T lymphocytes was observed in leukaemic cows (Fig. 2a). Lower numbers of CD2+, CD4+ and WC1-N2 T lymphocytes were recorded in heterozygotes than in homozygotes, however, these differences were statistically significant only for CD4+ and WC1-N2 T lymphocytes (P≤0.05). Similar differentiation between the genotypes caused by BLV infection was also observed for the percentage of the described cells (Figs 1b, 2b). A higher percentage of CD2, CD4 and CD8 T lymphocytes was recorded in clinically healthy heterozygotes than in α-LA^A/A and α-LA^B/B homozygotes, however, these differences were statistically significant only for CD4+ and CD8+ T lymphocytes (Fig. 1b). On the other hand, the percentage of the described cells in leukaemic cows varied differently. It involved a lower percentage of CD2+, CD4+, CD8+, and WC1-N2 T lymphocytes in heterozygotes than in α-LA^A/A and α-LA^B/B homozygotes, however, the differences between the genotypes were statistically significant only for T CD4+ lymphocytes (Fig. 2b).

While analysing the diversity of the peripheral blood lymphocyte profile in the first months of lactation, certain tendencies of changes among the CD19+ B lymphocytes and the CD2+ and CD4+ T lymphocytes were found (Table 1). The largest numbers of these cells were recorded in the second month and they decreased in the third month after calving. The differences between the analysed months of lactation, however, were not statistically significant. Moreover, a different tendency of changes in the particular months of lactation related with the BLV infection was observed (Figs 3a, 3b, 4a, 4b).

The greatest numbers of the CD19+ B lymphocytes and the CD2+, CD4+, CD8+, WC1-N2 T lymphocytes were registered in the leukaemic cows in the second month of lactation (Fig. 4a), however, these differences were statistically significant only in the case of the absolute number of the CD2+, CD4+, and the WC1-N2 T lymphocytes (P≤0.05) (Figs 4a, 4b).

The changes in the peripheral blood lymphocytes observed in the clinically healthy cows were not as regular as in the leukaemic animals (Figs 3a, 3b). A significant (P≤0.01) increase in the percentage of the CD19+ B lymphocytes in subsequent months after calving, as well as a similar direction of changes in the absolute number of these cells was reported. On the other hand, changes in the T lymphocytes in these cows through the entire lactation trimester were slight. The exception was the CD4+ T lymphocyte subpopulation whose smallest numbers were found in the first month of lactation, while the size of this subpopulation in the remaining months of lactation was maintained at a greater and constant level (Figs 3a, 4a). However, the differences between the first and the other two subsequent months of lactation were not statistically significant.

**Discussion**

In the presented study, a relation between the α-LA gene polymorphism and the peripheral blood lymphocyte profile, as well as an effect of the interaction between the α-LA gene polymorphism and the BLV infection on the T and the CD19+ B lymphocyte populations were observed. A CD19 molecule occurs on the surface of all B cells irrespective of the stage of their development.
Table 1
Analysed factors and peripheral blood lymphocyte profile in the examined cows

<table>
<thead>
<tr>
<th>Cell surface marker</th>
<th>Statistical measures</th>
<th>Genotypes of α-LA (1)</th>
<th>Result of diagnostic test (2)</th>
<th>Months of lactation (3)</th>
<th>Interaction</th>
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<td></td>
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<td>AA</td>
<td>AB</td>
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<td>BB-B2 (CD19) (%)</td>
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x Statistical calculations were made on logarithmic values (log10). Mean values denoted with the same small letter are significantly different at P ≤ 0.05. Mean values denoted with the same capital letter are significantly different at P ≤ 0.01. 1 x 2: the interaction of α-LA gene polymorphism and result of diagnostic test; 2 x 3: the interaction of result of diagnostic test and lactation months; * P≤0.05; ** P≤0.01
Fig. 1. Peripheral blood lymphocyte profile in cows of different α-LA genotypes; (mean±SD). Values denoted with the same small letter are significantly different at P≤0.05. Values denoted with the same capital letter are significantly different at P≤0.01.

Fig. 2. Peripheral blood lymphocyte profile in cows of different α-lactalbumin genotypes; (mean±SD). Values denoted with the same small letter are significantly different at P≤0.05. Values denoted with the same capital letter are significantly different at P≤0.01.
**Fig. 3.** Diversification of the peripheral blood lymphocyte profile in cows in the first months of lactation; (mean±SD). Values denoted with the same small letter are significantly different at $P \leq 0.05$.

**Fig. 4.** Diversification of the peripheral blood lymphocyte profile in cows in the first months of lactation; (mean±SD). Values denoted with the same small letter are significantly different at $P \leq 0.05$. 
After transformation of B lymphocyte into a plasmatic cell, this molecule undergoes atrophy (37). It plays an important role in the proliferation of B lymphocytes and is recognized as a key component of an intercellular pathway responsible for the signalling of cell activation (13, 19). In addition, it participates in the co-stimulation of B lymphocyte in the first step of cellular activation (12).

The CD2 molecule, occurring on the surface of the majority of the T cells, also plays a significant role in the co-stimulation of the lymphocytes (7). In the complex with the LFA-3 (CD58) ligand, it controls the development of thymocytes in the thymus and participates in activation of the mature T lymphocytes at the initial stage of the cell activation (9, 14). The CD2+ T lymphocytes are represented by helper lymphocytes (T_h) with the CD4 marker and by cytotoxic/suppressor (T_c/s) T lymphocytes with the CD8 surface marker. The CD4+ T lymphocytes support the humoral and cell mediated immune response as the result of direct contact or through the secreted cytokines. They are activated by antigens present in combination with the MHC molecules of class II. As the result of the contact with the B lymphocytes presenting antigen, these lymphocytes secret lymphokines which stimulate activation, proliferation and differentiation of the B lymphocytes into plasmatic cells. The T CD8+ lymphocytes are able to recognize viral, bacterial or neoplastic antigens presented by MHC class I molecules, and destroy them by secreting cytotoxic substances. The released cytokines also stimulate other cells to produce factors cytotoxic to infected or pathological cells (25, 35). Among the T lymphocytes, cells with γδ receptor can also be found. On their surface they do not have either CD4 or CD8 molecules (the CD4+ CD8+ double negative T lymphocytes) and their surface marker is WC1-N2 molecule (34). They exhibit the capacity of spontaneous cytotoxicity towards neoplastic cells and antibody-dependent cellular cytotoxicity (26). They can destroy both the microbe-infected and neoplastic cells as well as participate in the immunological response through the secreted cytokines such as IFN-γ (2).

A greater number and percentage of CD2+, CD4+, and CD8+ T cells recorded in clinically healthy α-LA<sup>A/B</sup> heterozygotes than in the other genotypes could indicate a relationship between the α-LA gene polymorphism and the population of these cells in cattle. The observed relations could also be the cause of the greater number of CD19+ B cells in α-LA<sup>A/B</sup> heterozygotes whose proliferation could be the effect of CD4+ T activity. Moreover, the more varied differentiation of the number and percentage of T lymphocytes recorded in α-LA<sup>A/B</sup> genotype leukemic animals than in clinically healthy animals of the same genotype seems to indicate a suppressive effect of BLV proteins on the expression of the α-LA immunological function and, consequently, on the restriction of T lymphocyte (mainly CD4+) proliferation. However, due to a small number of α-LA<sup>B/B</sup> homozygotes, formulation of unequivocal conclusions would be premature.

In the presented study, a diversification in the number and percentage of CD19+ B lymphocytes was also observed between the clinically healthy and the leukemic cows, however, the differences between these two groups were statistically significant only in relation to the number of these cells. Based on the previous studies (22), a significantly greater number and percentage of the CD19+ B lymphocytes were observed in the leukemic cows than in the clinically healthy cows. Similar results were reported by other authors (20, 36). Considering the young age of the examined cows, it seems that a smaller diversification of the percentage of the CD19+ B lymphocytes between the EBL+ and EBL- cows recorded in the present study may be related to an early developmental stage of the disease and to aleukaemic form of leukaemia frequently accompanied by B lymphocyte lymphopenia in some cows (3). Moreover, in the presented study a significantly lower percentage of the CD2+, CD4+, and CD8+ T cells was recorded in the EBL-positive than in the clinically healthy cows. Similar trends were reported in the previous study (22) and by other authors (3, 42). It can be presumed that it is the result of an increased proliferation of the B lymphocytes and an effect of viral proteins on the T lymphocyte sub-populations playing a vital role in the immunological response. The mechanism of BLV-induced lymphocytosis has not been identified yet. The progression of BLV infection to persistent lymphocytosis is likely to be linked with a reduced expression of classical Th1 and Th2 cytokines by CD4+ T cells, which can indicate aberrant Th regulation in BLV-infected animals (1). It is postulated that positive feedback loop between IL-2 and viral expression and the increased T-lymphocyte expression of IL-2 in BLV-infected cows contributes to development and/or maintenance of persistent B lymphocytosis (30, 38).

In the presented investigation, a diversification of the peripheral blood lymphocyte profile in the post-calving period was found. The smallest number of the CD19+ B lymphocytes and the CD4+ and CD8+ T lymphocytes was recorded in the second month after calving, however, the differences between the particular lactation months were not statistically significant. A similar trend was found in the previous study (22). Moreover, a different trend of changes in the peripheral blood lymphocyte profile related to the BLV infection was observed. Leukaemic cows had different numbers of B and T lymphocytes, while among clinically healthy cows, significant differences were found only for CD19+ B lymphocytes. Quantitative changes within T lymphocytes were small. The CD4+ T lymphocyte population was an exception. Their smallest number was recorded in the first month of lactation, however, the differences between this and the other months were statistically insignificant. Similar dynamics of changes in CD19+ B and CD4+ T lymphocytes related to BLV infection in cows in the first three months after calving was also observed in the previous studies (22), however, these results cannot be compared with the results of other authors since they concern different time frames and different lactation periods.
The differentiation of the peripheral blood lymphocyte profile in the postparturition period is certainly caused by physiological changes during calving and the initial period of lactation (28, 33). The relationships between the nervous, hormonal and immunological systems may be the reason for quantitative changes occurring within immune cells (27). Based on the study results, these interactions are likely to be modified by BLV which affects the B and T lymphocyte proliferation.

In conclusion, the \(\alpha\)-LA gene polymorphism exhibits correlations with the peripheral blood lymphocyte profile. They concern mainly CD4+ and CD8+ T lymphocytes. Moreover, a significant effect of BLV infection on the number of CD19+ B lymphocytes and percentage of CD2+, CD4+, and CD8+ T lymphocytes was recorded. The effect of the \(\alpha\)-LA gene polymorphism and BLV infection on the profile of the peripheral blood lymphocytes is modified by the interaction of these two factors. This interaction is exhibited by a different profile of changes among the EBL+ and EBL- animals. BLV also modifies the dynamics of changes in the percentage of CD19+ B lymphocytes in the first three months after calving.

The obtained results encourage the continuation of studies on the role of the \(\alpha\)-LA in the immunological processes and the pathogenesis of enzootic bovine leukosis.

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