PROFILE OF PERIPHERAL BLOOD LYMPHOCYTES IN THE FIRST MONTHS OF LIFE OF HEIFERS ORIGINATING FROM A LEUKAEMIC HERD

EWA KACZMARCZYK1, ANNA FIEDOROWICZ1, BARBARA BOJAROJ-NOŚOWICZ1 AND MARIUSZ MAJEWSKI2

1Department of Animal Genetics, Faculty of Animal Bioengineering, University of Warmia and Mazury, 10-719 Olsztyn, Poland
2Division of Clinical Physiology, Department of Functional Morphology, Faculty of Veterinary Medicine, University of Warmia and Mazury, 10-718 Olsztyn, Poland
e-mail: ewagen@uwm.edu.pl

Received for publication May 18, 2004.

Abstract

The objective of the present study was to determine the dynamics of changes in the profile of peripheral blood lymphocytes of healthy and leukemic heifers between the 3rd and 7th month of life. Enzootic bovine leukosis (EBL) was diagnosed with ELISA and PCR tests. Use was made of antiserum monoclonal antibodies (Mab), and conjugates of FITC and R-PE with an indirect immunofluorescence reaction (IMF). The present study revealed that approximately 62% of all examined heifers (40 out of 64 animals included in this investigation) were infected with bovine leukaemia virus (BLV). The most numerous subset of peripheral blood lymphocytes, observed in these animals was composed of CD19+ B-lymphocytes (39.14 ± 10.03%; mean ± SD), while the CD2+ and CD4+ positive T-lymphocytes were somewhat less numerous (18.72 ± 6.94% and 18.81 ± 6.70%, respectively). The subpopulations of CD8+ and WC1-N2-positive white blood cells were most scarce, constituting 8.39 ± 3.38% and 8.90 ± 5.46% of all lymphocytes, respectively. The ratio of CD4+ and CD8+ T lymphocytes was estimated as 2.13 ± 0.99. A significant difference in the total number and relative frequency of CD19+ B lymphocytes, as well as in the number of T lymphocytes with CD2+, CD4+ and WC1-N2 markers was observed between EBL-positive and -negative heifer calves. The relative frequency of CD19+ B lymphocytes subsets was also significantly different in particular age groups: the lowest number of those cells was found in the youngest animals, while the highest number was observed in the eldest individuals. A similar tendency was observed with regard to CD2+, CD4+ and CD8+ T lymphocytes, however, differences between particular age groups were not statistically significant.

Key words: heifers, enzootic bovine leukemia, lymphocyte subpopulations.

The infectious factor in enzootic bovine leukemia (EBL) is the bovine leukemia virus (BLV) belonging to type C oncogenous retroviruses (30). It shows a similar-
**Material and Methods**

**Animals and EBL diagnosis.** The experiment involved 64 Black-and-White heifer calves originating from one herd. Blood to be analysed was sampled from the jugular vein using heparin as an anticoagulant. EBL was diagnosed with the immuno-enzymatic ELISA kit (Rhône-Poulenc, France) and a PCR molecular test. The tests were performed when the heifers were three, four, five and seven month old.

**DNA isolation.** Genomic DNA was isolated from blood leukocytes obtained as a result of erythrocyte lysis with 0.85% solution of NH₄Cl. To isolate DNA, a Wizard Genomic DNA Purification Kit was used following the isolation procedure outlined by the producer (Promega, USA).

**PCR protocol.** A fragment of BLV genome with a length of 273 bp were amplified from genomic DNA (an indicator of a proper course of the PCR reaction). The earlier described PCR protocol (5) was used with starters, synthetised by the MWG-Biotech company (Germany), and the following nucleotide sequence:

- kapp1: 5'GAAATCCCTACCATCAATACC3'
- kapp2: 5'CCATCTACGCTAGTATGATG3'
- leu1: 5'GTGCCAACCTTCCCCGACGG3'
- leu2: 5'GACAGTCTGTTCCAAATGG3'
- FRG, working dilution of 0.1 µg/50µl (described below) for 30 min at 4°C.

**Immunofluorescent-staining.** Relative frequency of particular subsets of the lymphocytes studied was established by means of a routine single immunolabelling. Approximately 1.5x10⁷ of white blood cells were diluted in phosphate buffered saline (PBS), pH 7.4 depleted of Ca²⁺ and Mg²⁺ ions (Biomed, Lublin, PL), containing 1% bovine serum albumin (BSA) and 0.02% natrium azide and incubated with particular antiserum (described below) for 30 min at 4°C. Following, mouse monoclonal antibodies (mAb) (in a working dilution of 0.5 µg/50 µl each), against bovine antigens, were used: anti-B-B2 (binds to molecule similar to human CD19; BAQ44A; [13, 23]), anti-BoCD2 (MUC2A; [7]), anti-BoCD4 (GC50A1; [6]), anti-BoCD8 (CACT80C; [23]) and anti-WC1-N2 (BAQ4A, [6]) all purchased from VMDR Inc. Pullman, USA. Afterwards, the cell suspension was centrifuged (at 200 g), the resulting pellet was re-suspended and washed (5 min) and then centrifuged again. This procedure was repeated three times. After the last wash, the suspension was then incubated with FITC- or R-PE-conjugated goat anti-mouse IgG (Medac, FRG, working dilution of 0.1 µg/50 µl each) for 30 min at 4°C in order to visualize the primary antibody-antigen complex. After a final wash, a cellular smear has been prepared, cover-slipped with buffered glycerol (pH 8.4) and viewed and photographed under a Olympus BX51 microscope equipped with an epi-illuminator and appropriate filters sets.

The control was prepared with the same protocol but without the addition of the primary antibody.

**Counting of lymphocytes and statistical analysis of data.** Counting was based on 200 cells registered in the visual field. Calculations were made to determine the absolute number (total number of leukocytes (WBC) x % of cells with a specified phenotype CD /100) and percentage of lymphocytes with a specified surface marker.

The criterion of the conformity of the calculated parameters with a normal distribution model was used with reference to the analysed traits. In the absence of conformity with that model, the values of traits analysed were subjected to logarithmic transformation and statistical calculations were made on logarithmic values (log₁₀). Two-factor analysis of variance (ANOVA/MANOVA) and Duncan’s test of significance were applied. Differences between groups were verified at P ≤ 0.05 and P ≤ 0.01. Calculations were made with STATISTICA 6.0 computer software.

**Results**

The diagnostic tests found 40 heifers (62.50%) to be infected with enzootic bovine leukaemia virus and 24 heifers (37.50%) to be EBL-negative.

The largest population of peripheral blood lymphocytes was composed of cells with the CD1⁹ marker (5.04±2.37x10⁹/L; 39.14±10.03%). The CD2⁰ and CD4⁰ T lymphocytes were recorded frequently (2.31±0.98x10⁹/L; 18.72±6.94% and 2.31±1.05x10⁹/L; 18.81±6.70%, respectively), whereas CD8⁰ T lymphocytes and those with WC1-N2 marker were the most rare (1.04±0.47x10⁹/L; 8.39±3.38% and 1.12±0.78x10⁹/L; 8.90±5.46%, respectively). The ratio of CD4⁰ to CD8⁰ T lymphocytes accounted for 2.13±0.99 on average.

In analysing the diversification of the subpopulation numbers of peripheral blood lymphocytes in the examined animals, a significant effect of EBL was found on the number (P ≤ 0.01) and percentage (P ≤ 0.05) of CD1⁹ B lymphocytes. A higher number of B lymphocytes was identified in EBL-positive animals than in healthy ones (Figs 1a, 1b.). In addition, a significantly higher (P ≤ 0.05) absolute number of CD2⁰, CD4⁰ T lymphocytes and cells with WC1-N2 marker was reported in EBL-positive animals than in EBL-negative heifers (Fig. 1a).

A reverse direction of changes was observed with reference to the percentage of those cells (Fig. 1b). Lower values were reported in EBL-positive animals, whereas higher values were found in the healthy individuals. The differences between those groups of animals were not, however, confirmed statistically. No significant differences were observed either in the ratio of CD4/CD8 T lymphocytes between the compared groups of heifers (Fig. 3).

The analysis of the dynamics of changes in the subpopulations profiles of peripheral blood lymphocytes in subsequent months of life of the examined heifer calves indicated a significant (P ≤0.01) age-dependent increase in both the number and the percentage of CD1⁹ B lymphocytes (Figs 1a, 1b.).
The lowest number of those cells was reported for the youngest heifers (4.55x10^9/L; 36.68%), and the highest number for the eldest animals (5.82 x10^9/L; 43.31%). A similar tendency was observed with reference to the numbers of CD2^+ and CD4^+ T lymphocytes. Differences between the youngest (2.19x10^9/L and 1.83x10^9/L, respectively) and the eldest heifers (2.53x10^9/L and 2.25x10^9/L, respectively) were not confirmed statistically (P=0.07). In analysing the differentiation in the percentage of CD4^+ and CD8^+ T cells, the highest values were observed in the 5th month of life, and the lowest ones were in the 3rd and 4th month of life. The differences observed were not, however, confirmed statistically (P=0.07 and P=0.08, respectively) (Fig. 2b). No significant effect of the age of the calves on the value of the CD4/CD8 T lymphocyte ratio was observed (Fig. 4).
Fig. 2b. Population profile of peripheral blood lymphocytes in subsequent months of life of heifers (mean ± SD). Mean values denoted with different capital letters are significant at P ≤0.01.

Fig. 3. The incidence of EBL and the ratio of CD4/CD8 T lymphocyte subpopulation (mean ± SD).

Fig. 4. The ratio of CD4/CD8 T lymphocyte subsets in subsequent months of life (mean ± SD).

**Discussion**

In the presented study, a significant difference in the total number and the relative frequency of CD19⁺ B lymphocytes was observed between leukaemic and healthy heifer calves (Figs 1a, 1b). Higher numbers of those cells occurred in EBL-positive individuals, whereas lower numbers occurred in healthy ones. EBL is characterized by excessive proliferation of B lymphocytes (9, 31). In cattle (heifers, cows) with chronic lymphocytosis, the percentage of B lymphocytes has been reported to range from 30.90% to 58.77% (16, 29). A relatively low percentage of those cells observed in the presented study may indicate a low progression of pathologic changes in the heifers examined. Persistent lymphocytosis is also accompanied by a lower percentage of T lymphocytes and a higher ratio of CD4 to CD8 T lymphocytes, compared to healthy animals (14, 19, 29, 31). The EBL-positive individuals also demonstrated a higher percentage of T lymphocytes with TCR γδ (2, 24). In the presented study, the percentage of CD2⁺, CD4⁺ and CD8⁺ T lymphocytes was lower in EBL-positive animals compared to EBL-negative ones, however, statistically significant differences were not confirmed. A lower percentage of T lymphocytes in EBL-positive animals may point to a weakening of cellular-type resistance occurring in the initial and further stage of infection with BLV. A significant role is ascribed to
CD4+ T lymphocytes in recognizing proteins of the virus. Dysfunction of those cells is likely to enhance the progression of a BLV infection (20). A comparative analysis of leukaemic and healthy heifers in the presented study also revealed a slightly higher percentage of T lymphocytes with TCR\(\gamma\delta\) (WC1') and a slightly higher CD4/CD8 T lymphocyte ratio. Still, the differences were not confirmed statistically. On the other hand, the EBL-positive heifers demonstrated a significantly higher absolute number of T lymphocytes with CD2, CD4, and WC1-N2 markers.

The results obtained by other authors are not unequivocal. In animals with persistent lymphocytosis, some of them have reported a higher number of cells occurring within the analysed populations and sub-populations of peripheral blood lymphocytes (14, 26, 31), whereas others showed their lower levels (9, 25), or did not observe any differences between healthy and leukaemic animals (28). Those discrepancies may be of different origin, i.e. often scanty experimental material, various methods used to diagnose EBL, producing different detection efficiency of BLV infections, and different stage of disease progression. The results obtained in the current study point to the occurrence of general tendencies consistent with the results of some researchers (14, 26, 31).

The population number of CD19+ B lymphocytes was also significantly differentiated by the age of the examined heifer calves. The lowest and the highest numbers of the cells were reported in the youngest and eldest animals, respectively. A similar tendency of changes was observed with reference to CD2+, CD4+, and CD8+ T lymphocytes, however, the differences were not confirmed statistically (Figs 2a, 2b.). Investigations performed on cattle by other authors indicated the lowest numbers of B lymphocytes in foetuses, in the final stage of their development (3) as well as in calves, in the first hours and days after birth. In addition, an increase in the population number of those lymphocytes was observed along with the age of the calves (8, 17, 19). The low number of those cells in neonates is explained by a negative effect of maternal immunoglobulins on the proliferation of foetal B lymphocytes (3). When those immunoglobulins occur later in the blood plasma of neonates, they are also likely to inhibit the proliferation of those cells (17). An increment in the population number of those cells in the examined heifer calves was also accompanied by an elevated subpopulation number of CD4+ T lymphocytes; the differences were, however, not confirmed statistically (P=0.07). It is claimed that a higher number of CD4+ T lymphocytes reflects a strengthening of cellular and humoral resistance which in turn results from secretion of cytokines by those cells (20, 27). The observed changes may also be connected with immunological resistance gained by the organism. Quantitative changes occurring within T lymphocytes and reported in young cattle by other authors are not unequivocal and papers referring to that problem are still fragmentary. In calves, from birth up to the 3rd-9th week of life, Menge et al. (17) showed significantly more CD2+, CD4+, and CD8+ T lymphocytes and WC1+ cells, but did not observe any differences in the percentage of those cells. Opposite results were reported by Ayoub and Yang (1) in calves in the first 6 months of life. They did not observe any significant quantitative changes within a population and sub-population of T lymphocytes. On the other hand, Wilson et al. (32) revealed significantly more CD2+ T lymphocytes and significantly less CD4+, CD8+ T lymphocytes and WC1+ cells from the birth up to 3-5 months of life of the animals. It seems that those discrepancies may result mainly from both scanty experimental material and the different ages of the analysed animals.

Differentiation in the sub-populations numbers of peripheral blood lymphocytes observed in heifers in the presented research as well as in the experiments of other authors is probably caused by physiological changes in the hormonal profile referring mainly to the growth and maturation of heifers (33). In the animal organism there are interlinks between the nervous, hormonal and immunological systems. Due to that the cells of the immune system are the target cells for numerous hormones, neuropeptides, and substances produced by the immune cells themselves (cytokines and thymus hormones) affect the structure and functions of the nervous system and endocrine glands (10, 12). Those relationships may be the reason for quantitative changes occurring within immune cells in cattle in the period of their growth and development. They may also be the cause of the high individual variability in the peripheral blood lymphocyte profile in growing and developing heifers. The high variability of the number of CD19+ B lymphocytes in the examined animals may be additionally connected with the progression of BLV infection.

In conclusion, the obtained results seem to indicate the effect of natural infection with bovine leukaemia virus on the population number of CD19+ B lymphocytes in heifer calves aged 3-7 months. In addition, the young age of those animals was the reason for quantitative changes in the profile of peripheral blood lymphocytes. Significant changes were reported for CD19+ B lymphocytes, whereas lower differentiation was observed in the case of CD4+ and CD8+ T lymphocytes.

Acknowledgments: The authors thank to the staff of “GOSPOL” farm in Krzyżanowo, particularly Leszek Grenda and Jan Kasprzykowski for providing the research material.

References


