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PhD THESIS

CONTRIBUTIONS TO THE INVOLVEMENT OF IMMUNOLOGICAL MECHANISMS IN BOVINE MALIGNANT LEUKOCYTOSIS

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SUMMARY

Bovine malignant leukocytosis (EBL – enzootic bovine leukosis) is an infectious disease caused by a delta retrovirus from the category of type C oncoviruses, called a bovine leukaemia virus (BLV). A majority of previous researches have proven that a third of the infected animals have a form of pre-neoplastic disease – persistent lymphocytosis (PL) which is characterized by a relatively stable increase of the number of B lymphocytes in peripheral blood, and only 5% of these animals develop the neoplastic form of the disease, characterized by acute lymphocytic leukaemia or lymphoma/lymphosarcoma (through the infiltration of various organs with tumorally transformed B lymphocytes)

A group of researchers from the university of Berkley, California, coordinated by G.C.Buehring, in a study made between 1994-2005, showed that BLV can not only target blood cells, but also epithelial cells in the mammary tissue, an aspect that was demonstrated by techniques of immunohistochemistry and PCR on samples of mammary tissue from cows infected with BLV, as well as on samples of normal and malignant mammary tissue from women with and without breast cancer. The frequency of BLV in mammary tissue from women diagnosed with breast cancer was higher than in the samples from healthy women. Therefore, researches regarding the induction of malignant transformation by BLV are of a major interest in the present, in veterinary as well as in human medicine.

Present researches on leukaemia in humans and leukemic complexes in different species of animals are oriented towards deciphering the molecular mechanisms of virally induced cancer, and leukaemia respectively, on the one hand, as well as towards the ways of immune anti-tumour reactivity of the host organism, on the other.

The researches cited in literature and the latest scientific information in online databases have revealed the necessity for filling in some of the unknown facts on the pathophysiologic mechanism of enzootic bovine leukosis by correlating the immune changes in the blood with those that reflect energetic deficits and toxic-metabolic diseases of different organs.

These things considered, the purpose of this paper is to research the cellular and humoral immunitary status of bovines diagnosed serologically with BLV, so as to establish the main pathophysiological mechanism of leukosis in cattle, and to fill in some unknown facts regarding the pathophysiological disorders in the bovine leukemic complex.

The objectives of this study are: the epidemiological study of enzootic bovine leucosis in the herds of cattle from Vaslui county using serological tests based on the reaction between antigen-antibody (ID, ELISA); revealing the main pathophysiological mechanism involved in bovine malignant leukocytosis, through quantitative and qualitative haematological assessment of the erythrocytes, leucocytes and platelets and by biochemical investigation of the protein, energetic, hepatic, renal and mineral profiles.

In order to reach these objectives, we have done 427.394 serological examinations to detect EBL, and we examined 70 samples of blood from a haematological and biochemical point of view, revealing cellular and humoral immunological changes. The blood samples came from adult bovines (6-13 years) which were grouped in seropositive and seronegative lots for BLV, with or without persistent lymphocytosis, as follows: BLV+PL+ (marked or moderate lymphocytosis), BLV+PL- (no lymphocytosis), BLV- (reference lot).

The serological techniques used to detect BLV infection were: agar gel immunodiffusion (ID or AGID) and immune enzyme test (ELISA), according to the methodology described in official instructions (OIE Manual 2000). Haematological and biochemical parameters were determined with automatic and semi automatic analyzers which are available in veterinary laboratories: the automatic haematological analyzer MS4-5, the biochemical semi automatic analyzer EOS 880 Plus and specific kits. Cytological blood examination was performed on blood smears coloured with the classical method *May-Grünwald-Giemsa* (MGG). Statistical analysis of the results was made using the program SPSS 16.0 for Windows.

The results we obtained in our study are presented below.

The results of the epidemiological study made in a period of 6 years in Vaslui county show that 1643 of the 427.394 serological tests made for the detection of EBL (350.182 ID exams and 77.212 ID and ELISA exams) were positive. The incidence of the disease has continuously increased from 0,64 % at the beginning of the period to 0,22 % at the end of the studied period. Cartograms regarding the special distribution of EBL have shown an increase of the number of disease-free cities from 21 to 62. The degree of extension of the disease has decreased thanks to the improvement of prophylaxis measures (assanation by extraction, depopulation) doubled by the initiation of the national registering (identification) program, aimed at limiting the circulation of sick animals.

Malignant leukocytosis (leukosis) in bovines has a dominant pathochemical pathophysiological mechanism, with a starting point in the immune system of the organism, the manifestation being triggered by the specific virus. This statement is confirmed by the changes in

specific cellular and humoral immunity seen in the early stages of the infection, changes that are revealed by serological testing. The serological diagnosis test itself, based on the titre of antibodies, demonstrates the involvement of humoral immunity specific to the disease.

The results regarding the variation of erythrocyte constants showed lower values for RBC, Hb, PCV and MCHC in BLV+ cattle than in those BLV- (reference lot), a tendency towards a state of normocytic, hypochromic anaemia being obvious beginning with the early stages of the disease (in the asymptomatic BLV+ lot). In chronic diseases, and malignant states, including enzootic bovine leukaemia, there can be an association of a decrease in the production of Erythropoietin, medullar depression, haemolysis, chronic iron loss, the production of toxic substances because of metabolism disorders, all these factors leading to anaemia and cachexia of the animals.

The variations of leukocyte constants have allowed us to divide the investigated animals into groups, leukaemic and aleukemic, with severe lymphocytosis, reduced lymphocytosis and no lymphocytosis. In numerous studies, the haematological examination showed a moderate leukocytosis (from about 10.000 leucocytes/ μ l, to values over 100.000 leucocytes/ μ l). Haemograms obtained from cows with EBL with lymphoma demonstrate a wide variation of the blood leukocyte formula.

In the present study, in seropositive cows for BLV, the total number of leukocytes in peripheral blood varied from normal values – below 12.000/ μ l (variation limits of 5,51-11,81 $\times 10^3$ / μ l) to moderately high values – between 12.000 and 20.000/ μ l (variation limits of 14,40-18,73 $\times 10^3$ / μ l) and values of over 20.000/ μ l, without however surpassing 50.000/ μ l (variation limit of 24,62-34,20 $\times 10^3$ / μ l). Lot A, in which the average number of leucocytes is higher than 20.000/ μ l and lot B, with a total leukocyte average over 12.000/ μ l, present a severe leukocytosis, whereas lot C, with a total leukocyte average under 12.000/ μ l, present no leukocytosis. This classification shows that, according to serological and haematological exams, most of the animals were in the first stage of the disease, that of asymptomatic carrier, but in different phases of the interaction between the virus and the immune system of the host organism. We include here lots B and C, that sum 20 of the 29 investigated animals (68,96 %). The remaining 9 animals, belonging to group A (31,04 %) were in a more advanced stage of the disease, characterised by a marked leukocytosis caused by an accumulation of leukocytes in the blood, a phenomenon in the evolution of EBL known as persistent lymphocytosis –PL (in some studies this is called chronic leukocytic leukaemia – CLL). We haven't encountered in our study the final stage of the disease, that of tumour development (lymphoma or lymphosarcoma).

The lymphocytosis that characterises, according to literature, 20-30 % of the cows with enzootic bovine leucosis in the first stage of evolution, was seen in the present study in 14 of the 29 serologically positive cows, which represents a percentage of 48,27 %.

The increase in the number of lymphocytes and the presence of a basophilic cytoplasm of lymphocytes and cells similar to immune cells and plasmacytes, indicate a chronic antigenic stimulation and an increase in the synthesis of immunoglobulins, as in the case of diseases that have a long period of latency.

In the present study, the number of lymphocytes in BLV+ animals, considered to be in the stage of persistent lymphocytosis, varied between 7,6 and 25,4 thousands/ μ l, while the reference lot have an average number of $3,8 \pm 0,28$ thousands/ μ l lymphocytes. The cows in this group (BLV⁺PL⁺) also had neutrophilia (with an absolute number of neutrophils of 4,9-7,9 thousands/ μ l, higher than the average value for the reference BLV negative lot, of $4,55 \pm 0,78$ thousands/ μ l) and monocytosis (the limits of variation for the number of monocytes between 0,6-2,3 thousands/ μ l, higher than the average for the reference lot, $0,4 \pm 0,01$ thousands/ μ l).

Well known data in scientific literature specify that the profile of cytokines involved in the activation of the immune system, changes with the progression of various chronic diseases, including enzootic bovine leukosis, these cytokines being extremely variable according to the stage of the disease. Apart from T and B lymphocytes, the neutrophils and the monocyte-macrophage system have an essential role in the production of interleukins (D.Pyeon and G.A. Splitter, 1998). Furthermore, it has been shown that, although the major target for BLV is the B lymphocyte, there are other cells, mainly the monocyte-macrophage lineage, where the bovine leucosis virus could persist for long periods of time (N. Gillet et al., 2007).

The presence of a greater number of monocytes in peripheral blood, especially in animals with persistent lymphocytosis, could be explained by a decrease of programmed cellular death of these cells, as a consequence on the integration of the virus in the genome of the host cell, but also by the increase in the production of the cells involved in the change of the cytokine profile corresponding to the progression of the disease.

By examining microscopically blood smears coloured MGG, lymphocytes were differentiated morphologically and functionally according to their size (large, medium and small Lf) and according to the presence or absence of intracytoplasmatic granulations (Lf with granulations – reactive Lf, and without granulations – nonreactive Lf).

The results of morphological examination of lymphocytes have shown a lower percentage of large lymphocytes in BLV positive lots (A, B, C) as compared to the BLV negative reference

lot which had the highest percentage of large lymphocytes. Average percentage and absolute values of the number of small lymphocytes show differences between the BLV positive lots, directly proportional to the degree of lymphocytosis registered. Bovines with severe lymphocytosis have the highest number of small lymphocytes (an average of $10,59 \pm 5,66$, representing $74,93 \pm 20,35$ %), while bovines with a low degree of lymphocytosis have a number of small lymphocytes of $3,70 \pm 1,39$, representing $56,45 \pm 18,79$ %, and bovines without lymphocytosis have a number of small Lf of $2,06 \pm 0,69$, representing $59,42 \pm 16,95$ %.

Lymphocytic reactivity has shown differences between the investigated lots, the lowest percentage of reactive lymphocytes being seen in bovines from lot A – $4,74 \pm 3,48$ %, while lots B and C, as well as the reference lot, had a percentage of reactive lymphocytes within the reference limits cited in literature: lot B – $12,59 \pm 5,86$ %, lot C - $8,89 \pm 5,08$ %, reference lot - $10,23 \pm 0,71$ %, which shows the fact that severe lymphocytosis is the result of the proliferation of transformed, immunologically non reactive lymphocytes.

The variations of platelet constants depend, as do leukocyte constants, on the evolution stage of the disease. The average number of platelets had the lowest values in lot B (with a low degree of lymphocytosis – $344,2 \pm 97,93$ thousands/ μ l), without, however, decreasing below the inferior limit of the species. If in the asymptomatic stage of the disease, the number of platelets doesn't differ considerably from that of the reference lot, BLV negative, in the transition stage towards persistent lymphocytosis, as it is the case for lot B in our study, thrombocytopenia was seen, and in a more advanced stage, of persistent lymphocytosis, although the average number of platelets is close to that of the reference lot, there are wide variations from thrombocytopenia to thrombocytosis. Viruses, including leukemic ones, can induce in certain stages of the disease medullar hypoplasia, resulting in aleukemic leukaemia, which can be associated with thrombocytopenia and anaemia.

The results regarding the variation of biochemical parameters in EBL have shown a series of changes, some of them never having been cited in literature.

Humoral immunity specific to the immunological mechanism has revealed changes in the protein profile. Total protein in the serum of BLV+ cows of $9,63 \pm 0,97$ g/dl, is higher than that seen in the reference, BLV- lot, and are much higher than the average value of reference. Serum alpha globulin as well as beta globulin have shown slight increases in the BLV positive lot, that were not statistically significant ($1,35 \pm 0,96$ g/dl, and $1,60 \pm 0,97$ g/dl respectively), as compared to the values in the reference lot ($1,09 \pm 0,20$ g/dl, and $1,30 \pm 0,32$ g/dl respectively). Serum gamma globulins have shown a statistically significant increase – $3,43 \pm 1,06$ g/dl in positive

cows, as compared to the reference negative lot - $1,92 \pm 0,26$ g/dl. These changes are similar to the results of other studies that explain the increase in gamma globulins by a tumoral proliferation of B lymphocytes.

The results regarding the investigation of the energetic profile have shown some variation in BLV positive cows and we consider that they are an addition to scientific literature. We have obtained a value of $59,5 \pm 17,92$ mg/dl for serum triglycerides in BLV+ cows that was twice the average for the reference BLV- lot, the increase of the level of serum cholesterol ($159,2 \pm 37,82$ mg/dl) as compared to the known reference value (100 mg/dl); a net increase in BLV+ cows of lactic acid level ($40,02 \pm 6,56$ mg/dl), an aspect that was shown by most prior studies on humans and animals with leukemias.

Hypercholesterolemia is a controversy in literature, some authors mentioning an increase in cholesterol levels in precancerous states of the disease, and others mentioning hypocholesterolemia in stages of advanced malignancy of the haematopoietic system. An explanation for the increase in the level of lactic acid is the increase of its production by leukemic cells (which have a disturbed function of the mitochondria responsible for aerobic energetic metabolism)

The researches regarding the hepatic and renal profile we obtained can be considered original for bovine leukosis. A serum GGT level ($24,38 \pm 9,75$ U/l) at the upper reference limit, a PAL level ($124 \pm 80,32$ U/l) higher than the reference values and a BUN value ($32,08 \pm 3,57$ mg/dl) higher than the reference value of the species, demonstrates that in BLV+ cows, hepatic and renal cellular disorders appear in the early stages of the disease, as a result of leukemic lymphocytic infiltration of the liver and kidneys.

The mineral profile investigated in this study shows in BLV+ cows a serum concentration of calcium ($9,91 \pm 0,26$ mg/dl) and magnesium ($2,22 \pm 0,23$ mg/dl) within the normal range, but a decrease of the concentration of inorganic phosphorus ($2,98 \pm 1,06$ mg/dl), an aspect that was not previously mentioned in scientific literature.

In conclusion, the presence of anti-BLV antibodies shown by serological testing, blood cell modifications (leukocytes, platelets and erythrocytes) revealed through haematological quantitative and qualitative examination, as well as the biochemical changes of the protein, energetic, hepatic, renal and mineral profiles in the cows we investigated in the present study, show the involvement of complex immunological and pathochemical mechanisms in the development of bovine enzootic leucosis.